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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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ABSTRACT

Background

The aim of this study was to investigate the role of the upstream kinase TAK1 and the canonical NF- κ B pathway colorectal in cancer (CRC). Immunohistochemistry was used to assess the expression of TAK1/pTAK1 and canonical NF- κ B 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 IKK β 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 IKK β 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 IKK β was significantly associated with decreased CSS and with markers of the tumour microenvironment. These results support the hypothesis that NF- κ B 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

1.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 (Hanahan & 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 & 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 & 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, Park *et al.*, 2014b).

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, 2015),(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- κ B) pathway. The NF- κ B family of transcription factors functions via two main pathways – the canonical and the non-canonical pathways (Perkins & Gilmore, 2006). In the canonical

pathway the IKK complex, which is composed of the catalytic subunits IKK α and IKK β and the regulatory scaffold protein NEMO, is maintained in the cytoplasm. On stimulation of the pathway by cytokines such as TNF α or IL-1, the complex is activated and phosphorylates I κ B which leads to the release of p50/RelA which then translocates to the nucleus (Perkins & 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- κ B pathway (Sakurai *et al.*, 1998, Sakurai *et al.*, 1999). Interestingly TAK1 has also been associated with a possible third NF- κ B pathway which is independent of the controls recognised in the other two pathways (Margalef *et al.*, 2012, Margalef *et al.*, 2015).

The aim of this study was to investigate the role of components of the canonical NF- κ B 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.

2.Methods

2.1Patients

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 $\leq 10\text{mg/L}$ were given a score of 0, patients with CRP $> 10\text{mg/L}$ and albumin $\geq 35\text{g/L}$ a score of 1 and patients with CRP $> 10\text{mg/ml}$ and albumin $< 35\text{g/L}$ 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.*, 2015) were used to determine the pre-operative inflammatory state. A NLR > 5 was considered elevated; patients with a platelet count $< 400 \times 10^9/\text{L}$ and a neutrophil count $< 7.5 \times 10^9/\text{L}$ were given a score of 0, either a neutrophil count $> 7.5 \times 10^9/\text{L}$ or a platelet count $> 400 \times 10^9/\text{L}$ a score of 1 and those with both an elevated neutrophil and platelet count a score of 2.

2.2 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, Richards *et al.*, 2014) (Park, Richards, *et al.*, 2014). If the TSP was $\leq 50\%$ 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.

2.3 Assessment of the expression of members of the canonical NF- κ B pathway

Immunohistochemistry was performed to assess protein levels of TAK1, TAK1 phosphorylated at threonine 187(pTAK1), IKK β , p65 (RelA) and phosphorylated p65, using a colorectal cancer TMA of 242 patients (Figure 1). Prior to this, antibody specificity was confirmed using western blotting to identify a single band of the predicted molecular weight (Supplementary Figures 1, 2 & 3). TMA sections (2.5 μ m) 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 pH9 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, IKK β (Abcam ab32135) at 1:500, IKK α (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 EnVisionTM (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 orJQ], 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.

2.4 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 β 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- κ B pathway was examined using the Chi-square test for linear trend. The relationship between cytoplasmic pTAK1, cytoplasmic IKK β and cytoplasmic pTAK1/IKK β 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- κ B 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.

3.Results

The characteristics of the 242 patients within the cohort are shown in Table 1. Briefly, 59.5% were 65 or 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 ($P=0.032$) (Figure 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 associated with decreased cancer-specific survival when compared to low and high expression ($P=0.032$) (Figure 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) (Figure 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).

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) (Figure 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 IKK β 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 IKK β was associated with cancer-specific survival, high expression was associated with poor outcome when compared to low and moderate expression (P=0.046) (Figure 2b). Mean cancer-specific survival for patients expressing low levels of IKK β 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 IKK β expression. When 5 year survival data was considered, 51% of patients expressing high levels of cytoplasmic IKK β , were alive at 5 years, compared to 59% of patients with moderate expression and 71% of patients whose tumours expressed low levels of IKK β . When this was assessed across tumour state 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 IKK β 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 IKK β and Klintrup-Makinen grade (P=0.015) and a direct linear relationship was observed between IKK β Glasgow Microenvironment Score (P=0.029) and an indirect relationship was observed between IKK β and Neutrophil-Lymphocyte ratio (P=0.03). There was no significant relationship observed between any of the other characteristics.

Association between cytoplasmic IKK β expression and cancer-specific survival was then investigated in different subgroups of colorectal cancer patients. Cytoplasmic IKK β 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 P=0.067, Mutated P=0.264).

The relationship between clinicopathological factors, the tumour microenvironment, systemic inflammatory responses, pTAK1, IKK β 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 IKK β (HR 1.75,95%CI 1.05-2.91, P=0.033) (Table 2).

4. 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 IKK β was significantly associated with decreased survival and with markers of the tumour microenvironment. The present study demonstrates that members of the canonical NF- κ B 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 IKK β was associated with decreased cancer-specific survival and with the tumour microenvironment but not with *BRAF* or MSI status. On multivariate analysis only cytoplasmic IKK β 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 hypothesise that this might be due to the fact the TAK 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 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 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- κ B pathway and has been recognised as having a role in the activation of the canonical NF- κ B pathway (Sakurai *et al.*, 1998, Sakurai *et al.*, 1999). It has been reported to activate the I κ B kinase (IKK) complex which is composed of IKK α , IKK β 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 TNF α (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 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 IKK β in colitis-associated cancer has previously been reported (Greten *et al.*, 2004). Using mouse models, deletion of IKK β in intestinal epithelial cells leads to a decrease in tumour size but not a reduction in inflammation (Greten *et al.*, 2004). A role for IKK β 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 IKK β in mouse intestinal epithelial cells induces tumours. This supports our observation that elevated cytoplasmic IKK β expression is associated with a reduced cancer-specific survival.

In conclusion the current study investigated the role of components of the canonical NF- κ B 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 IKK β in the cytoplasm of CRC cells may have some prognostic value, however this requires validation in a larger independent cohort.

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Figure Legends

Figure 1 Representative images of IKK β 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 (x100 magnification). Image at x400 demonstrating cytoplasmic and nuclear expression patterns (q).

Figure 2 The relationship between cytoplasmic pTAK1 and cytoplasmic IKK β expression and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer: (a) cytoplasmic pTAK1 expression (P=0.032) and (b) cytoplasmic IKK β expression (P=0.046).

Figure 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: (a) cytoplasmic pTAK1 stratified with wildtype *BRAF* expression (P=0.014) and (b) cytoplasmic pTAK1 stratified with mutated *BRAF* expression (P=0.105).

Supplementary Figure Legends

Figure 1. Antibody specificity determined for TAK1 and p-TAK1 using cell lysates prepared from cells stimulated with TNF α (20ng/ml) over a time course of 120 minutes. Tubulin was used as a loading control.

Figure 2. Antibody specificity determined for IKK β using lysates prepared from cells transfected with si RNA. Tubulin used as loading control.

Figure 3. Antibody specificity determined for p65 and p-p65 using lysates prepared from cell stimulated with TNF α (20ng/ml) and IL-1 (20ng/ml) over a time course of 120 minutes. Tubulin was used as a loading control.

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)

Table 2: Univariate and Multivariate analysis of members of the Canonical NF- κ B 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 IKK β	1.47(1.08-2.01)	0.046	1.75(1.05-2.91)	0.033
Nuclear IKK β	1.03(0.76-1.40)	0.648		
Cytoplasmic IKK α	0.96(0.58-1.60)	0.877		
Nuclear IKK α	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		

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)	
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)	7(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

Table 4: Relationship between cytoplasmic IKK β 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)				
	46(24.7)	49(26.3)	52(28.0)	0.030
≤5	20(10.8)	10(5.4)	9(4.8)	
>5				
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)	

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

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

Biomarker	Univariate	P-value	Multivariate	P-value
	HR (95% CI)		HR (95% CI)	
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 ($\leq 7.5 \times 10^9/L$ / $> 7.5 \times 10^9/L$)	1.84(1.00-3.39)	0.047		
Platelet count ($\leq 400 \times 10^9/L$ / $> 400 \times 10^9/L$)	1.909(0.85-4.20)	0.110		
NPS (0/1/2)	1.98(1.19-3.28)	0.022		

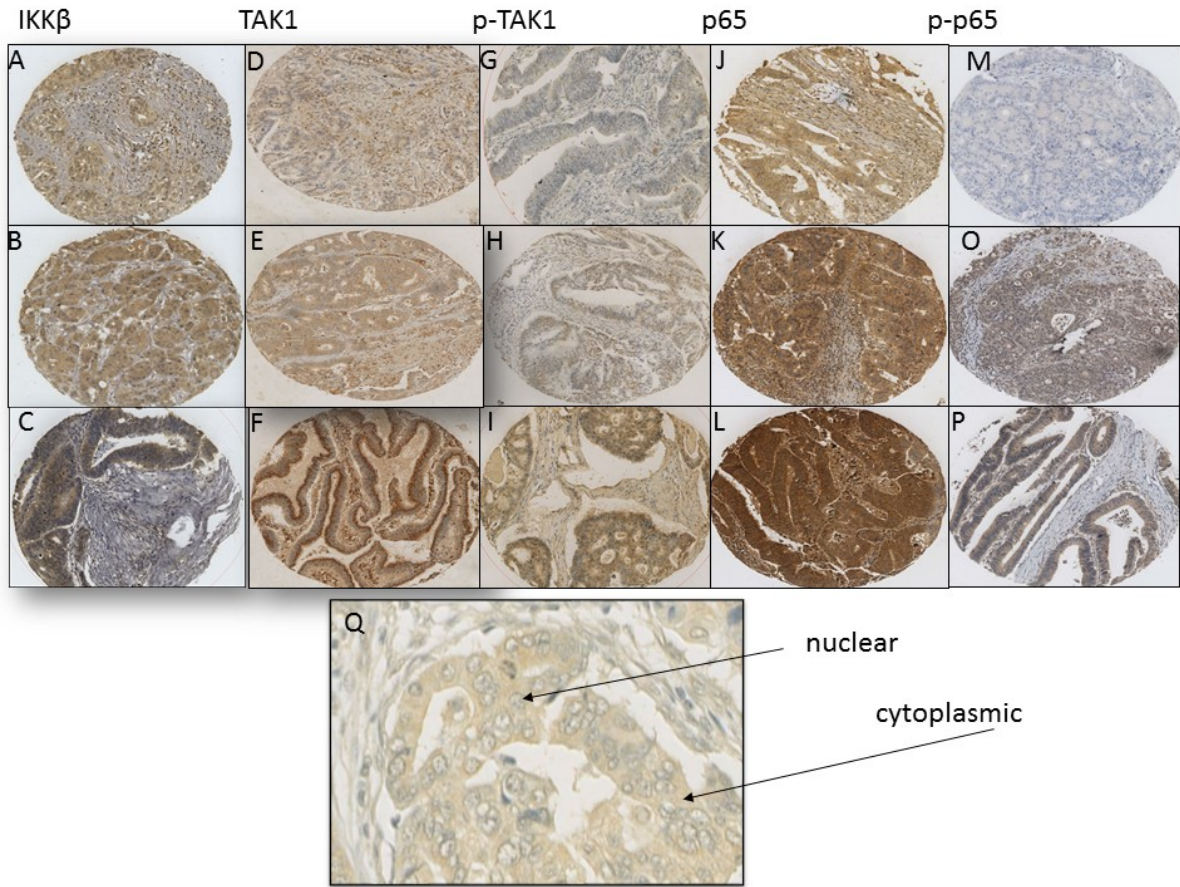


Figure 1

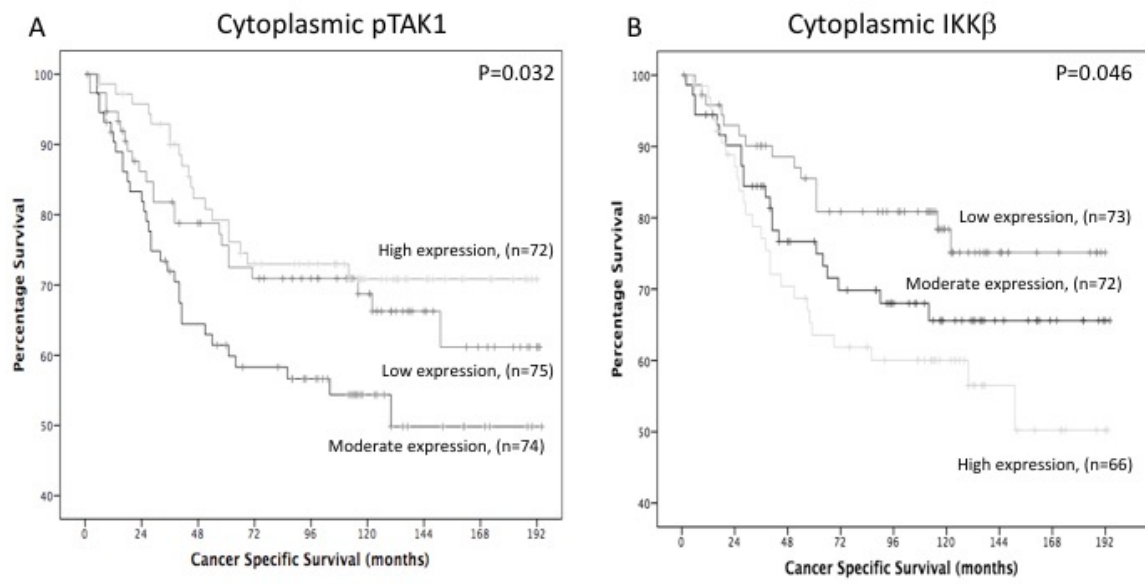


Figure 2

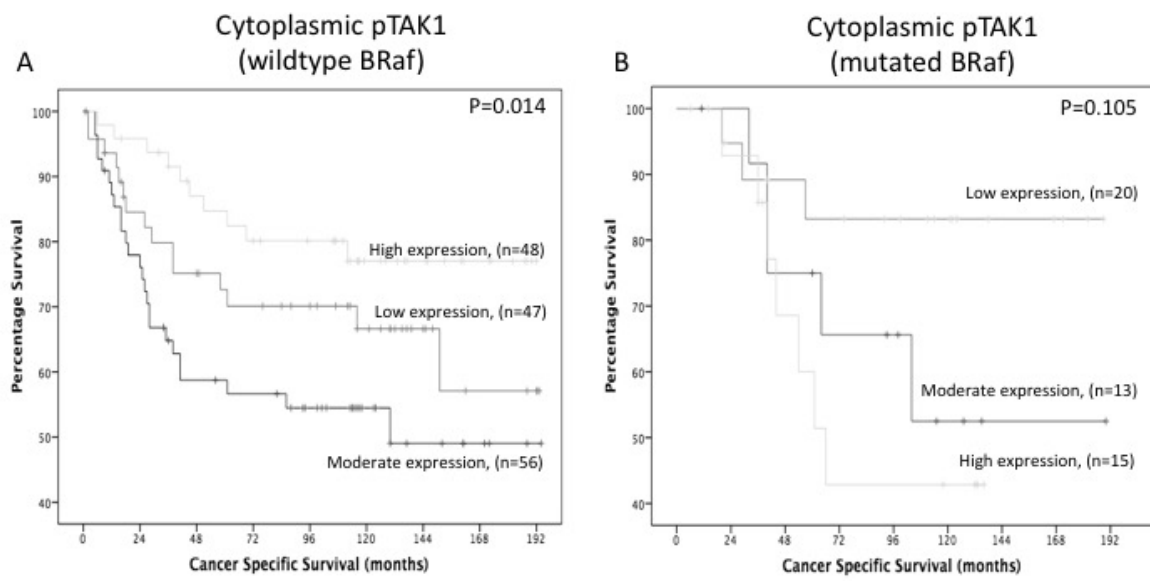
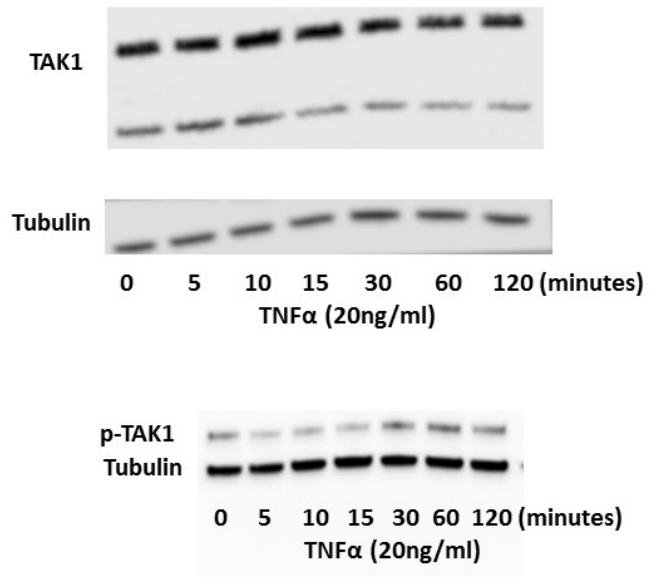
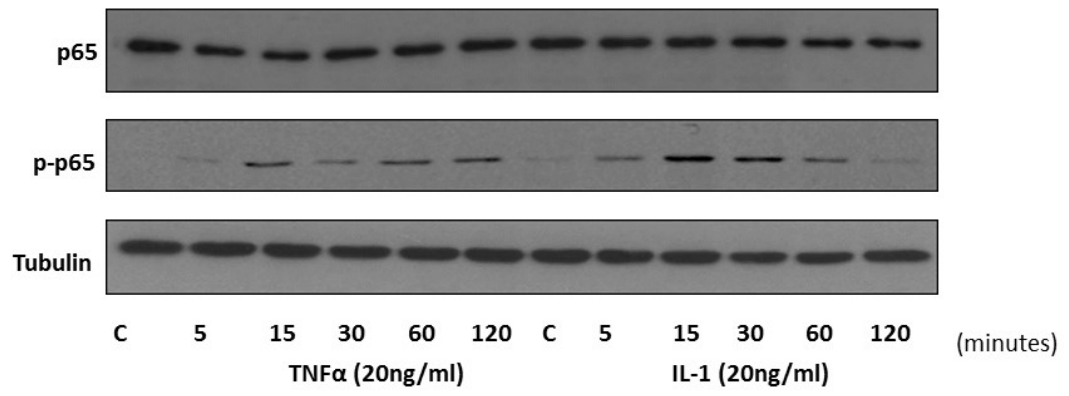


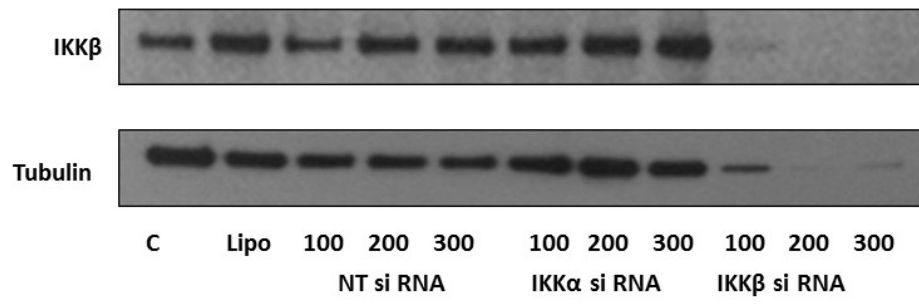
Figure 3



Sup Figure 1



Sup Figure 2



Sup Figure 3