NF-kB pathways in the development and progression of Colorectal Cancer

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Abstract

NF-κB has been widely implicated in the development and progression of cancer. In colorectal cancer, NF-κB has a key role in cancer-related processes such as cell proliferation, apoptosis, angiogenesis and metastasis. The role of NF-κB in CRC is complex, owed to the crosstalk with other signalling pathways. Whilst there is sufficient evidence gained from cell lines and animal models that NF-κB is involved in cancer related processes, due to a lack of studies in human tissue, the clinical evidence of its importance is limited in patients with CRC. This review will summarise evidence relating to how NF-κB is involved in the development and progression of CRC and comment on future work to be carried out.

Introduction

Worldwide, colorectal cancer (CRC) is the third most common cause of cancer-related death (1). The majority of CRC cases are sporadic with a smaller proportion resulting as a consequence of inflammatory bowel disease (IBD) or inherited germ line mutations. The relationship between the NF-κB pathway, inflammation and cancer has been widely reviewed (2–4). Much of the initial research linking NF-κB and CRC focused on chronic inflammation, was performed in mouse models of colitis and importantly demonstrated NF-κB functions are cell-type and tumour-type specific (5,6). Few reviews to date have evaluated the clinical significance of studies investigating NF-κB in CRC. NF-κB activation supports tumourigenesis by enhancing cell proliferation and angiogenesis, inhibiting apoptosis and promoting cell invasion and metastasis (4). There are two distinct but interacting arms of the NF-κB pathway: canonical (activated by TNF-α, Toll-like receptor ligands e.g. lipopolysaccharide (LPS), and IL-1) and non-canonical (activated by TNF superfamily members BAFF, CD40, receptor-activated NF-κB ligand (RANKL) and lymphotoxin β) (7–12). NF-κB is a surrogate for a family of five related Rel proteins: RelA/p65, c-Rel, RelB, NF-κB1 (p50, p105 or p50/p105) or NF-κB 2 (p52, p100 or p52/100). p50 and p52 are produced by processing of precursors p105 and p100 respectively. These proteins have a common 300 amino acid Rel homology domain (RHD) (4) therefore once activated, their structural similarities allow formation of homo- and heterodimers, nuclear localisation, DNA binding and association with IκB inhibitors. In the latent cell IκB inhibitory proteins (IκBα, IκBβ, IκBε, IκBγ, IκBζ, p100 and p105) remain bound to the DD (dimerization domain) and NLS (nuclear localisation signal) of NF-κB proteins which are consequently rendered inactive within the cytoplasm (5–9). IκBs contain multiple copies of a 30-33 amino acid sequence called ankyrin repeats which mediate their interaction with the RHD of NF-κB proteins, masking their NLS and thereby holding them bound inactive within the cytoplasm. Key steps in both arms of the pathway include activation by IκB kinase (IKK) complex resulting in the

phosphorylation-induced proteasomal degradation of IκB proteins, allowing dimers to form, enter the nucleus and bind to κB sites in promotor or enhancer regions of target genes (4,10). Each arm of the pathway has key roles in innate and adaptive immune responses, cell survival, cell death and inflammation (3).

Sequential genetic mutations give rise to sporadic CRC through the adenoma-carcinoma sequence. Mutation of the adenomatous polyposis coli (APC) gene (and subsequent WNT activation and stabilisation of β-catenin) is recognised as one of the primary steps in colorectal adenoma formation. No activating mutations of NF-κB in CRC have been reported (3) however, constitutive activation of NF-κB has been observed (13–16) and is associated with higher tumour stage (15,17), treatment resistance (14,18–21) and poor survival outcomes (22). As activation of NF-κB pathways have been associated with poor prognosis it is possible that members of the NF-κB pathways could be employed as prognostic markers or indeed novel therapeutic targets for CRC. Importantly, the role of NF-κB in the transition of pre-malignant polyp to invasive carcinoma is unclear.

The role of inflammation in CRC is undisputed and has been studied at the local tumour and systemic level. Within the field of CRC research there have been significant developments in understanding the role of the tumour microenvironment; stromal invasion and the local immune response are well recognised as important prognostic features (23,24). Consensus Molecular Subtypes and phenotypic subtypes of CRC have identified distinct groups of patients with strong immune activation, this has been associated with improved prognosis (25,26). Specifically, understanding the immune cell varieties and signalling pathways regulating them have resulted in development of prognostic biomarkers and subsequent introduction of immunotherapies in patients with metastatic disease. The addition of targeted immune therapies against EGFR (epidermal growth factor receptor), VEGFR (vascular endothelial growth factor receptor) and immune check points, to standard chemotherapy regimes, have led to improved survival in patients with metastatic CRC cancer, and have rendered some patients suitable for metastectomy with the potential for cure. NF-κB has key roles in a range of cancer-related processes and clearly presents itself as a potential prognostic biomarker and therapeutic target in CRC. This review will summarise evidence between 1995 and 2017 that reports on the relationship between NF-κB, colitis-associated however primarily sporadic CRC and how this signalling pathway influences the clinical development and progression of CRC.

Proliferation and evading apoptosis/antigrowth signalling

Colorectal polys

Polyps are recognised as precursors to CRC. They are classified into either adenomas or serrated/hyperplastic polyps, both with malignant potential. Vogelstein and colleagues have detailed the stepwise accumulation of molecular alterations that accompany the adenoma-carcinoma sequence (chromosomal instability pathway) which sees transformation through increasing grades of adenomatous dysplasia to the development of invasive carcinoma. (27). The role of NF-κB in adenoma formation in the context of colitis-associated cancer has been studied in mouse models that suggest NF-κB is implicated in the early stages of adenoma formation. However, there are no known studies that have mapped NF-κB expression in human tissue with respect to adenoma-carcinoma transformation. Nonetheless, a hybrid mouse model with intestinal epithelial cell-specific allelic deletion of APC and constitutive expression of IKKβ, displayed increase colonic adenoma formation than their APC allele deleted only counterpart. This study reported the hybrid model expressing IKKβ mostly increased adenoma number but did not increase the size of the adenoma suggesting IKKβ is implicated in adenoma initiation or early establishment (28). Serrated/hyperplastic polyps are considered as developing via distinct mechanism to that described by Vogelstein and it would be of interest to study NF-κB in this context. If confirmed in human studies NF-κB could be employed clinically identify which polyps have malignant potential.

The anti-apoptotic activity of NF-κB is mediated via Bcl2, Bcl-x_L, cFLIP, cIAP2, amongst other genes (4). Anti-apoptotic protein BAG-1 (Bcl-2-assocaited athanogene-1) is involved in key processes such as proliferation, cell signalling, transcription and apoptosis (29). Overexpression of BAG-1 has been reported in colorectal adenomas and carcinomas. The same study reported knockdown of BAG-1 in colon cancer cell lines inhibited NF-κB transcriptional activity (30). A separate study reported this process is regulated by antigrowth protein/tumour suppressor Rb (retinoblastoma) (31). Inhibition of NF-κB with BAG-1 siRNA/inhibitor of NF-κB suppressed cell yield and induced apoptosis. This study concluded inhibition of NF-κB and therefore suppression of BAG-1 represents a novel therapeutic strategy in CRC (30).

Colitis associated cancer

The first genetic evidence linking canonical NF-κB activity, inflammation and CRC was from a mouse model of colitis-associated cancer where deletion of IKKβ in intestinal epithelial cells (IECs) and myeloid cells had distinct outcomes; deletion of IKKβ in IECs showed reduced adenoma incidence which was not related to levels of inflammation but had a direct effect on tumour promotion, as demonstrated by reduction in anti-apoptotic Bcl-2 protein, Bcl-x_L. In contrast, deletion of IKKβ in myeloid cells was associated with a less marked reduction in adenoma incidence but was associated with reduced adenoma size, with reduced expression of genes encoding pro-inflammatory

cytokines such as IL-1β, IL-6 and TNFα, without no effect on apoptosis (5). These results suggest IKKβ mediated NF-κB activity has cell-specific roles in the development of colitis-associated cancer. Importantly, this study demonstrates the early role of NF-κB in the development of pre-cancerous adenomas which could be exploited clinically as a potential target. Therefore it is of interest to understand how NF-κB is implicated in the treatment of mouse models of colitis, IKKβ inhibitors have been observed to lead to smaller and less frequent tumour formation and may offer a novel mode of early intervention (33).

When reviewed as a whole this evidence supports the hypothesis that there is a relationship between inflammation, NF-κB activity and tumourigenesis and indeed that NF-κB has a role in directly promoting tumourigenesis in colitis-associated cancer.

Metastatic CRC

The relationship between NF-κB and inflammation induced tumourigenesis was investigated in a metastatic colon cancer mouse model where it was observed that injection with bacterial LPS resulted in metastatic tumour growth via inflammatory mediator TNF-α. Intraperitoneal injection with colon cells transfected with mutant IκBα reduced tumour burden and improved murine survival. In vitro, this inhibition of NF-κB resulted in a cytocidal effect mediated by TRAIL (TNF-related apoptosis-inducing ligand) (34). Observations from this study further support the link between NF-κB induced inflammation and growth of malignant cells in colon cancer.

Human colon cancer stem cells have been identified (35,36) and can promote tumour formation and metastases in vivo (37,38). Signalling pathways implicated in this process include IL-6/STAT3 (Signal Transduction Activator of Transcription-3) (37), activation of AMPK/mTOR and NF-κB (38). In a genetic model with restricted WNT-activation, ablation of NF-κB (p65) restricted intestinal crypt stem cell expansion. Moreover, enhanced NF-κB activity increased Wnt activation and induced dedifferentiation of non-stem cells that acquire tumour-initiating capacity (39). Altogether, these studies support the role of NF-κB and indeed other closely linked signalling pathways such as STAT3 in colon cancer stem cells implicated in tumourigenesis. Moreover, they highlight the need for further studies to investigate the effect of inhibiting NF-κB/STAT3 on tumour growth in the context of cancer stem cells to fully understand how these pathways could be exploited clinically as therapeutic targets.

Evasion of apoptosis is a hallmark of a cancer. When proliferating cancer cells encounter hostile conditions e.g. hypoxia or insufficiency in nutrients, there are regulatory mechanisms which kick in. These include cell cycle arrest, apoptosis and autophagy. These processes are under the influence of tumour suppressor genes such as p53 (40). The p53 gene is mutated in 40-60% of CRCs (41). Whilst mutation of p53 is considered an important step in colorectal pathogenesis, studies investigating its prognostic and predictive capacity have reported conflicting results (42). However, the relationship between NF-κB and p53 and its importance in the development and progression of cancer is well

documented (43). A drug- and cytokine-induced p53/NF-κB crosstalk has been reported in colorectal cancer cells. Furthermore, TNF-α induced NF-κB target genes in colorectal cancer cells are dependent on this p53/NF-κB interaction (44). NF-κB is essential in p53-mediated cell death (45,46), mutant p53 prolonged activation of NF-κB in cultured cells and was associated with increased susceptibility to colitis-associated CRC in vivo (47).

Fibronectin is an extracellular glycoprotein which plays a role in cell adhesion, growth, migration, differentiation, inflammatory cell activity and tumour angiogenesis (48–51) and is highly expressed in CRC cell lines. Silencing of fibronectin increased apoptosis-related gene products caspase-3, p53, PARP, Bax and cytochrome *c* in vivo but decreased levels of NF-κB suggesting a relationship between fibronectin and the NF-κB/p53 signalling pathway (52), again providing evidence that NF-κB plays a central role in regulating cell death in development and progression of CRC. Altogether, there is substantial evidence which shows NF-κB plays a diverse role in processes relating to proliferation, apoptosis and antigrowth signalling at all stages of CRC development i.e. from adenoma through to metastatic carcinoma and therefore offer exciting opportunities that could be exploited clinically.

Angiogenesis

Angiogenesis is a hallmark of cancer driven by VEGF (vascular endothelial growth factor). In addition to VEGF, CXCL1, CXCL8, IL-8 and COX-2 are also angiogenic regulators under the influence of NF-κB (53). Monoclonal antibodies against VEGF have been used to treat metastatic CRC for some time. VEGF is also inducible under hypoxic conditions by hypoxia-inducible factor (HIF). There are three HIF-α proteins: HIF-1α, HIF-2α and HIF-3α (regulator).The HIF pathway regulates cellular responses to hypoxia and also has a role in regulating inflammation and immune responses via cross-talk with NF-κB (54). The importance of this crosstalk was demonstrated by a group who confirmed HIF-1α inhibition by NF-κB inhibitor parthenolide which lead to downregulation of hypoxia-dependent angiogenesis in HUVECs (human umbilical vein endothelial cells), reduction in HIF-1α target gene proteins and inhibition of hypoxia induced epithelial-mesenchymal transition (EMT). Furthermore, parthenolide treatment inhibited tumour growth, angiogenesis and progression in CRC xenograft models (55). Knock down of NEMO in CRC cell lines resulted in greater TNFα induced apoptosis and reduced expression of angiogenic factors IL-8, growth-regulated alpha protein (Groα) and monocyte chemoattractant protein 1 (MCP-1). In vivo, this knockdown resulted in reduced angiogenesis, tumour volumes, serum and tumour IL-8 expression and improved tumour regression when treated with fluorouracil (13,56). In CRC tissue, expression of NF-κB (p65) associates directly with expression of HIF-1α, VEGF and histological evidence of vascular invasion (16,57).

These studies support the role of NF-κB, angiogenesis and tumour progression in CRC and highlight the importance of understanding crosstalk with other pathways to develop targeted therapies, particularly in those patients who may not respond to or experience toxicity with the available anti-VEGF therapy.

Metastasis and self-sufficiency in growth signals

Unsurprisingly, NF-κB is involved in EMT of tumour cells. EMT results in epithelial cells acquiring the invasive and metastatic properties of mesenchymal cells and thus important in promoting cancer metastasis (58).

Chemokines have been implicated in tumour metastases. Colon cells transfected with chemokine CXCL8 DNA displayed higher migration rate and EMT-like phenotype with increased expression of EMT markers N-cadherin, Vimentin and α-SMA as well as activation of PI3/Akt/NF-κB pathway. CXCL8 transfected mice displayed more rapid tumour growth compared to controls (59).

Olfactomedins are a family of proteins which mediate development of the nervous system and haematopoiesis. Olfactomedin 4 (OLFM4) is an intestinal stem cell marker and a target of the Wnt/β-catenin pathway. A recent study reported OLFM4 as a negative regulator of Wnt/β-catenin and NF-κB pathways with inhibition of colon cancer development in APC mutated mice (60). Olfactomedin 1 (OLFM1) also has a tumour suppressive role and is able to suppress growth, migration and invasion of CRC cells in vitro; Additionally, OLFM1 negatively regulated non-canonical NF-κB activity of NIK. Knockdown of OLFM1 promoted growth and metastasis of CRC cells in vivo; Low expression of OLFM1 in CRC tissue was associated with lymph node involvement, distant metastases and poor overall survival (61).

Matrix Metalloproteinases (MMPs) are a family of proteolytic enzymes which have a physiological role of tissue remodelling and as such are able to degrade the extracellular matrix and facilitate tumour invasion. Overexpression of MMP 1, 2, 3, 7, 9 and 13 has been observed in human CRC (62) and were associated with poor prognosis and metastasis (63). The expression of MMP-9 is regulated by several transcription factors including NF-κB (64,65). The mechanistic link between NF-κB and MMP-9 was studied in CRC cells lacking the β subunit of the IKK complex and this showed NF-κB (IKK activity and p65) is required for TNF-α induced MMP-9 gene expression (66).

NIK- and IKK-β-binding protein (NIBP) have been implicated in the regulation of cytokine-induced canonical NF-κB signalling. NIBP is over expressed in CRC tissue and is associated with metastasis (67,68). Nude mice injected with NIBP knockdown cells demonstrated less tumour formation, with no detectable tumour at 3 months (69). Expression of NIBP in 114 patient tissue samples of CRC was associated with tumour metastasis. The same study demonstrated NIBP overexpression induces activation of the canonical NF-κB pathway in vitro. Additionally, xenografts of NIBP-overexpressing

cells generated liver metastases with increased expression of p65, MMP-2 and MMP-9 suggesting NIBP may increase CRC metastases via canonical NF-κB activity and upregulation MMP-2 and MMP-9 (70).

Cytoskeletal proteins Fascin and Ezrin have also been implicated in EMT and metastasis via NF-κB activity (71,72).

Whilst the benefits of an adaptive immune response in the CRC tumour microenvironment has been widely demonstrated and now underpins therapeutic manipulation, the innate immune response is less understood. Specifically, the role of tumour-associated macrophages (TAMs) in CRC is controversial and the mechanisms behind phenotypical skewing towards either M1-like (pro-inflammatory/anti-tumour Th1 response) or M2-like (anti-inflammatory/pro-tumour Th2 response) in CRC is incompletely understood (73). In a study of knock in mice expressing a dominant negative form of IKK-α, treatment with carcinogen resulted in markedly reduced adenoma formation at 20 weeks, with smaller tumours and slower proliferation rates. This was associated with increased recruitment of M1 (tumouricidal) -like myeloid cells into the tumour, which was not cell-autonomous but depended on interaction between mutant IKK-α epithelial and immune cells (74). In an interesting study using a model of peritoneal metastasis in immune-competent mice, intraperitoneal injection with IκBα-suppressed colon cells induced an M1-like macrophage phenotype, with reduced liver and peritoneal metastases in vivo. This was associated with increased intratumoural activated $CD4^+$ and $CD8^+$ T cells and reduced angiogenesis (75). This study suggests targeting NF-κB could induce a phenotypic switch from M2-like immunosuppressive to an M1-anti-tumour macrophage in CRC.

Activin A is a member of the TGFβ superfamily and has been reported to have a role in inflammatory responses (76). Activin A is overexpressed in human colorectal tumours, especially in stage IV disease suggesting activin A may have a role in advanced colorectal cancer (77). Signalling pathways for activin and TGFβ are frequently disrupted in CRC (78). Activin down regulates p21 and increases migration and invasion of colon cancer cells (79). The link between NF-κB signalling and activin ligand expression was investigated by the same group who reported activin, but not TGFβ, induced NF-κB activation with subsequent increased MDM2 ubiquitin ligase and degradation of p21 via PI3K dependent mechanism. Further to this, a functional role for NF-κB in activin-induced colon cancer cell migration was reported (80).

Altogether, these findings further implicate NF-κB in important cancer processes such as EMT, autonomous growth signalling and regulation of the tumour microenvironment and how this might be manipulated as a therapeutic strategy.

NF-κB crosstalk with other signalling pathways

There is evidence of cross talk between canonical and non-canonical NF-κB pathways (81,82), NF-κB and a number of other pathways, as well as studies demonstrating the ability of IKK kinases to directly target substrates in an NF-κB independent manner. The IKKs are regulated via their phosphorylation sites. Once phosphorylated, conformational change of the kinase results in activation. IKK-β is phosphorylated at serines 177 and 181(83,84). Crosstalk of IKK-β with Src kinase family is demonstrated by the c-Src dependent phosphorylation of Tyr^{188} and Tyr^{199} near the activation loop of IKK-β (85). IKK-α is phosphorylated at serines 176 and 180, autophosphorylation occurs at these sites when part of the IKK-β/NEMO complex. With respect to the non-canonical NF-κB pathway, NIK phosphorylates IKKα at serine 176 (86). Other proteins are also regulated via IKKα activation. For example, one mechanism of crosstalk between PI3K-Akt and NF-κB pathways is via phosphorylation of Thr23 on IKK-α (87). Both IKK-α and IKKβ have an NLS which renders them able to directly phosphorylate proteins at the nuclear level (12). For example, IKK-α has been implicated in the induction of NF-κB gene expression via phosphorylation of Ser 10 in Histone 3 (88). It would be of interest to study the role/expression of IKK phosphorylation sites in CRC. There has been little success in the development of IKK inhibitors and focused targeting of phosphorylation sites may offer an alternative therapeutic strategy.

IL-1 can stimulate PI3K/Akt-dependent activation of NF-κB (p65), independent of the classical NF-κB pathway (89,90). In non-colon cells, oncogenic Ras required P13K and Akt to stimulate NF-κB-dependent transcription by targeting the transactivation domain of p65 rather than via classical degradation of IκB and subsequent translocation of p65 to the nucleus, with resulting reduced apoptosis (91). Loss of APC function and derangement of Wnt/β-catenin signalling have been firmly implicated in the development of CRC (92). It has been reported that IKK-α and IKKβ interacted with and were able to phosphorylate β-catenin, with IKK-α specifically able to increase β-catenin-dependent gene expression in colon cancer cell lines (93). A separate study reported constitutive IKK-β expression in mouse IEC induced spontaneous tumour formation, enhanced chemical- and APC mutation-mediated carcinogenesis and contrary to previous reports was associated with increased expression of β-catenin (94). Furthermore, in human CRC tissue, it has been reported that PI3K/Akt/IKK-α pathway regulates NF-κB and β-catenin with the ability to influence transcription of genes implicated in angiogenesis and metastasis (95). When this evidence is considered as a whole it strongly supports the hypothesis that there is an interaction between the β-catenin, PI3/AKT and NF-κB pathways in CRC development and progression. However, it is unclear whether IKKβ interacts with Wnt/B-catenin signalling in an NF-κB-dependent or independent fashion (96).

The NF-κB and STAT3 interaction is important in orchestrating the immune and inflammatory response within the tumour microenvironment and each has distinct functions in immune and cancer cells (97). NF-κB and STAT3 regulate common processes and share regulatory binding sites of anti-

apoptotic, cell cycle and proliferation, tissue resistance and repair genes, in addition to genes regulating angiogenesis/responses to hypoxia, chemokine and cytokine expression (98). As well as having distinct and overlapping target genes, as demonstrated by the pattern of their respective gene binding sites (99,100), NF-κB and STAT3 have been shown to physically interact (99,101) with STAT3 causing the nuclear retention of NF-κB; STAT3 can prolong nuclear retention of phosphorylated p65 (102). Co-localisation of STAT3 and NF-κB has been observed in CRC cell lines (103). MicroRNAs (miRNAs) function to suppress gene expression and have been reported to regulate NF-κB activity (104,105). In CRC cell lines transfected with microRNA mimics/inhibitors, miRNA-221 and miRNA-222 activated NF-κB (p65) and STAT3 with associated increase in miRNA-221 and miRNA-222 suggesting these mRNAs function in a positive feedback regulatory loop to increase NF-κB and STAT3 activity. STAT3 activation is mediated by the tyrosine kinase JAK1 as a results of cytokine activity e.g. Interluekin-6 (IL-6). IL-6 is produced in an NF-κB dependent manner within myeloid cells in a model of colitis-associated cancer. The pro-tumourigenic effects of IL-6 were mediated by STAT3 (106). STAT3 activity is found in epithelial and lymphocytic cells; inhibition of STAT3 in colon cancer cells curbs tumour cell proliferation in xenograft models of CRC (107). In CRC tissue, expression of STAT3 is associated with downregulation of the local adaptive immune response and decreased cancer-specific survival (108). No activating mutations in NF-κB or STAT3 have been detected in CRC suggesting they are secreted in a paracrine or autocrine fashion (109).

Adaptive immunity is important in cancer immunosurveillance (110) and in CRC, the presence of tumour infiltrating immune cells (Th1 derivatives) have shown prognostic value independent of the widely used UICC-TNM classification (23). In contrast, high expression of T helper cell 17 (Th17) cells in CRC is associated with poor prognosis (111). There are a number of studies based on inflammation-associated colon cancer models that implicate inflammatory cell-derived cytokines (IL-17A, IL-22, IL-6) to tumourigenesis (106,112,113). A study investigating the immune/inflammatory infiltrate and cytokine response in sporadic CRC found no differences in immune cell composition between uninvolved mucosa and tumour but did observe that this transition is marked by a functional switch in T cells leading to the accumulation of Th17-related cytokines, TNF-α and IL-6. Additionally, tumour-infiltrating lymphocyte-derived supernatant induced proliferation of colon cancer cell lines with activation and co-localisation of NF-κB and STAT3. These findings were confirmed in a mouse model of CRC. Interestingly, this study also observed that administration of a compound targeting STAT3/NF-κB activation and crosstalk, reduced levels of STAT3/NF-κB-activating cytokines and tumour growth (103). This study highlights the importance of understanding the relationship between immune cell subtypes and cytokines and their influence on intracellular signalling pathways in sporadic CRC as well as highlighting STAT3/NF-κB crosstalk as a novel

therapeutic target in CRC. Moreover, it highlights a deficiency in studies of NF-κB in models of sporadic CRC.

ERK5 and MEK5 overexpression has been reported in human adenomas and carcinomas and ERK5 expression correlated with NF-κB. Colon cells with over activated ERK5 displayed increased NF-κB nuclear translocation and transcriptional activity; orthoptically implanted tumours with over activated MEK5/ERK5 had greater lymph node metastasis, altogether suggesting MEK5/ERK/NF-κB signalling is implicated in tumourigenesis and metastasis (114).

As previously discussed the HIF pathway regulates cellular response to hypoxia and also have a role in regulating inflammation and immune responses via crosstalk with NF-κB. HIF and NF-κB share common activating stimuli, regulators and targets (54,115); Both HIF and NF-κB are activated in a TAK1-dependent manner (116) and IKK-β deficient mice demonstrate defective HIF expression and induction of target genes including vascular endothelial growth factor (VEGF) (115). High expression of HIF-2α in CRC tissue is associated with increasing tumour stage, poor tumour differentiation, lymphovascular invasion, COX-2 expression and worse overall survival (117).

NF-κB independent roles of IKK-α

The study of IKK-α in CRC has provided insights into its NF-κB independent functions and highlights it as an important player in colorectal carcinogenesis. Constitutive activation of IKK-α resulted in phosphorylation of SMRT at serine 2410 in CRC tissue. Furthermore, IKK-α binds to Notch-dependent gene promotors with resultant release of chromatin-bound SMRT and up-regulation of Notch-dependent gene and anti-apoptotic *cIAP2* upregulation (118). Inhibition of IKK-α restored SMRT chromatin binding with inhibition of Notch-dependent gene transcription and subsequent reduction in tumour size in a CRC xenograft model (118). The same group have reported a truncated isoform of IKK-α with the predicted molecular weight of 45kda was generated by cathepsin mediated cleavage of full length IKK-α (FL-IKK-α) within early endosomes. Nuclear truncated IKK-α (p45-IKK-α) forms a complex with full length IKK-α and NEMO and is responsible for regulating phosphorylation of SMRT and histone H3. This study provides evidence that p45-IKK-α results in diminished apoptosis in vitro and is required for CRC tumour growth in vivo (119). More recently, the same group demonstrated that KRAS and BRAF mutant ($BRAF^{V600E}$) cells are different; whilst KRAS was able to induce canonical NF-κB signalling, $BRAF^{V600E}$ did not have the same effect. $BRAF^{V600E}$ is required for p45-IKK-α phosphorylation in a TAK1 dependent but NF-κB independent fashion. Additionally, endosomal inhibition reduced proliferation of $BRAF^{V600E}$ cells in culture as well as tumour growth and metastasis in xenograft models of CRC. The addition of an endosomal inhibitor appeared to potentiate the effect of Irinotecan in this model. FL-IKK-α, NEMO, TAK1 and BRAF were associated with the endosomal compartment however the nature of this association is unclear (120).

Whilst there is sufficient evidence gained from cell line and animal work relating to crosstalk between IKK-α and other signalling pathways in the development and progression of CRC, there is limited evidence that this is clinically important due to lack of studies investigating NF-κB in patient specimens and how this may relate to phenotypic tumour characteristics and patient outcomes.

NF-κB and therapeutics

In 2005, Gilmore and Hersovitch reported over 750 inhibitors of the NF-κB pathway including a variety of natural and synthetic molecules (121).

COX-2 is a target gene of NF-κB (122) and is responsible for prostaglandin synthesis during inflammation. COX-2 is overexpressed in CRC (123), has been directly linked to colorectal tumourigenesis (124,125) and underpins the basis of numerous studies investigating the chemoprophylactic role of non-steroidal anti-inflammatory (NSAIDs) drugs such as aspirin and selective COX-2 inhibitors (126). The mechanism of COX-2 induction is not fully understood however it has been reported that in vitro inhibition of NF-κB can reduce COX-2 expression in colorectal cancer cells (127–129). Additionally, it has been reported that upregulation of COX-2 is associated with increased expression of p65, p50 and IKK-α in human colorectal cancer tissue, altogether suggesting a relationship between NF-κB signalling and COX-2 expression. Epidemiological data has demonstrated the protection conferred by Aspirin against CRC cancer (130). NF-κB plays a central role in anti-apoptotic NSAID activity in CRC cells in vitro (131–133). Both Aspirin and Sulindac inhibit IKK-β (131,134) and Aspirin, specifically, is able to induce phosphorylation and proteosomal-mediated degradation of IκBα and nuclear translocation of p65 (133). Although Aspirin is known as an inhibitor of NF-κB, in xenograft models of human CRC, Aspirin increased levels of phosphorylated IκBα and nuclear p65 with associated apoptotic effect (135). These apoptotic effects were also reported in colon cells in vitro, independent of p53 or MMR status (136). It is evident that part of the chemoprophylactic nature of Aspirin in CRC can be attributed to NF-κB activity.

Curcumin is a polyphenol derived from the spice turmeric and is active against a number of cell signalling pathways including NF-κB. In TNF stimulated cells treated with curcumin, onc study reported absence of NF-κB activity by inhibition of IκBα phosphorylation and translocation of p65 to the nucleus (137). In vitro, curcumin inhibited COX-2 by inhibition of NIK/IKK signalling and thus prevented phosphorylation of IκB in human colon cells (127). Curcumin has been and continues to be studied extensively. It has been reported to reduced colonic tumourigenesis in animal models (138,139) and to favour polarisation of macrophages toward an anti-tumour MI-like phenotype in xenograft models of colon cancer (139). NF-κB activity has been implicated in chemo- and radio-resistance (18,19) and curcumin has been reported to overcome this by blocking NF-κB activity (20). The combination of curcumin and chemotherapeutic agent capecitabine effectively reduced tumour

volume, proliferation and microvessel density in vivo when compared to controls, with associated suppression of NF-κB-regulated gene products (21). In a separate study, curcumin was able to overcome oxaliplatin resistance via inhibition of NF-κB, in vitro (127). Later, the efficacious combination of curcumin and oxaliplatin was observed in xenograft models of CRC (140). The same group are currently investigating the combination of curcumin with FOLFOX chemotherapy within a phase II clinical trial of patients with metastatic CRC (141).

For classical NF-κB activity, the proteasome plays an important role in degradation of IκB inhibitory protein. Proteosome inhibitor Bortezomib which is used to treat multiple myeloma has been investigated in CRC. Leucovorin is often given in combination with 5-fluorouracil (5-FU) to treat metastatic CRC. Treatment of CRC cells with leucovorin and Bortezomib enhanced caspase activity and apoptosis more effectively than either agent alone; these findings were confirmed in mouse CRC xenografts (142). Bortezomib has demonstrated limited efficacy in combination with standard treatment in clinical trials of patients with metastatic CRC (143,144). (See Table 1 for a summary of drugs targeting NF-κB that have clinical application in CRC).

NF-κB activity interferes with the efficacy of chemotherapeutic agents through induction of anti-apoptotic genes. There is no doubt that inhibiting IKK kinases suppresses CRC tumour growth in vivo as well as enhancing sensitivity to 5-FU (33,145,146). A number of IKK inhibitors have been reported in the literature but none have made it into clinical practice. Until now tools to unpick that exact nature of NF-κB inhibition have been limited due to the broad spectrum and non-specific nature of inhibitors. However, it has now been reported that first in class IKK-α specific inhibitors are available (147). This should enable IKK-α as a novel therapeutic target to be explored and if validated might lead to translation into patients.

NF-κB as a biomarker in CRC

Activation of NF-κB has been observed in response to chemo- and radiotherapy (148–150). Much of the data relating to NF-κB as a predictor of treatment resistance in CRC is preclinical (151–156). In these studies aberrant NF-κB activity has been implicated in resistance to drugs used in the treatment of CRC such as 5-FU, oxaliplatin and irinotecan. Irinotecan is used in the treatment of metastatic CRC, many patients will develop eventual treatment resistance and disease progression which has been attributed to irinotecan-mediated NF-κB activation (155). The addition of an inhibitor of IKK-α to mouse xenograft models of colon cancer potentiated the antitumoural effect of irinotecan and increased the sensitivity of colon cells to 5-FU in vitro (146). Within a clinical trial of patients with irinotecan-refractory metastatic CRC treated with cetuximab and irinotecan, patients whose tumours expressed NF-κB (p65) had inferior response rates and overall survival compared to those patients whose tumours did not express NF-κB (18). This is one of the only clinical studies which has aimed to evaluate NF-κB in a predictive capacity.

Overexpression of p65 in CRC tissue was associated with increasing tumour stage (15,17) and poor overall survival (157). A study investigating the prognostic significance of NF-κB, HIF-1α and VEGF expression in 148 patients who had undergone potentially curative resection for stage III CRC found that NF-κB expression was an independent predictor of overall survival. Additionally, the 56 patients who relapsed had inferior response rates (and overall survival) to palliative chemotherapy.

Patients with metastatic CRC undergo testing to determine KRAS status; this practice is widely established and harbouring the KRAS mutation precludes treatment with anti-epidermal growth factor (EGFR) receptor monoclonal antibody. NF-κB can be activated through RAS-RAF signalling pathway. Expression of oncogenic KRAS has been reported to result in NF-κB activation (158–160). Knockdown of KRAS reduces expression of p65 and phosphorylated-IκBα in CRC cell lines (161). Studies have reported higher expression of NF-κB (p65) in CRC tissue of patients with KRAS mutation than those without. NF-κB activation in the presence of KRAS mutation was associated with inferior overall survival in stage I-IV disease and reduced response to chemotherapy in patients with metastatic disease (162). However, in a study of patients with KRAS wild-type metastatic CRC treated with irinotecan and cetuximab, 65% had tumours expressing p65 and this was associated with poorer progression-free and overall survival than those patients with NF-κB negative tumours, suggesting NF-κB expression is prognostic irrespective of KRAS mutational status.

A study of 22 patients investigating the relationship between radiotherapy for rectal cancer, NF-κB activation and treatment response reported that NF-κB target genes are upregulated in response to a single fraction of radiotherapy however, whilst expression of NF-κB subunit p50 was prognostic for overall survival, it was not predictive of pathological response to radiotherapy.

Concluding remarks

This review summarises evidence from pre-clinical, translational and clinical studies that have reported on NF-κB with respect to the development and progression of CRC. There is substantial evidence implicating NF-κB in all stages of CRC development, from early adenoma to invasive cancer and metastasis. The role of NF-κB in CRC is undoubtedly complex and this complexity is enhanced by the crosstalk of NF-κB with a multitude of signalling pathways and regulators. Therefore, together with its essential role in normal cellular physiology it is unsurprising that inhibitors of NF-κB have yet to successfully emerge into clinical practice.

There is a growing body of evidence from studies in cell lines and animals implicating NF-κB activity in the development, progression and treatment resistance of CRC. Many studies focus on the measurement of p65 activity/expression as a measure of NF-κB activity which largely reflects the canonical pathway. Future studies should pursue investigation of non-canonical NF-κB activity which

appears to be underexplored. Importantly, there is a lack of studies in human tissue and therefore in understanding the relationship between NF-κB and phenotypic tumour characteristics. It would also be prudent to understand the role of NF-κB activity in the development of pre-malignant polyps and their transformation into invasive cancers. Finally, molecular and phenotypic subtypes of CRC have been proposed (25,26) and the study of NF-κB in the context of these subtypes would help to further decipher the biology of CRC.

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Name of drug/molecule	**Type of molecule i.e. natural product, synthetic, protein**	**Point of activity**	**References**	**Stage of development/Clinical application**
Aspirin, sodium salicylate	Synthetic	IκBα phosphorylation, IKKβ inhibition	Kopp & Ghosh, 1994 [1]; Yin et al. 1998 [2] Stark et al. 2005 [3]	Epidemiological evidence of reduced rick of colorectal adenomas with regular aspirin use[4–7]. Use of daily aspirin was associated with reduction in incidence of colorectal adenomas in patients with history of adenomas or previous colorectal cancer [8]. There are a number of active clinical trials aimed at determining the risk-benefit profile of Aspirin as a chemopreventive agent.
Selective COX-2 inhibitors e.g. Celecoxib, Rofecoxib	Synthetic	COX- dependent and independent activity	Grösch et al., 2006 [9]	Three large double blind randomised controlled trials showed COX-2 inhibitors prevent recurrence of sporadic adenomas with an increased cardiovascular risk [10–12]. Currently not routinely used as a preventative or therapeutic option in CRC.
Curcumin (Diferulolylmethane)	Natural antioxidant	NIK/IKK signalling IκBα phosphorylation, translocation of p65	Plummer et al., 1999 [13] Singh and Aggarwal, 1995 [14]	Phase I/II clinical trial of combination FOLFOX and curcumin in patients with metastatic colorectal cancer with inoperable liver metastases has completed recruitment phase – results awaited [15].
Bortezomib	Synthetic	Proteasome	Adams &	Limited efficacy of

			Kauffman, 2004 [16]	Bortezomib in combination with standard treatment in phase I/II clinical trials of patients with metastatic CRC [17,18].