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NF-κB2 and RELB offer prognostic information in colorectal cancer and *NFKB2* rs7897947 represents a genetic risk factor for disease development

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ABSTRACT

The Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family of transcription factors plays an important role in immune responses and cancer development and progression. We have focused on NF-κB2 and RELB of the alternative pathway of NF-κB, which remains largely underexplored in colorectal cancer (CRC). We found that NF-κB2 and RELB protein levels were upregulated in tumour and surrounding stromal tissue compared to distant non-neoplastic tissue (NN) and associated stroma ($p<0.001$ in all associations). Moreover, low RELB protein expression was associated with decreased overall survival ($p=0.032$). Lower RELB gene expression levels were observed in tumour compared to NN tissue ($p=0.003$) and were associated with shorter time to progression (TTP) ($p=0.025$). NF-κB2 gene expression levels were similar in tumour and NN tissue, but higher tumour levels were prognostic for improved survival ($p=0.038$) and TTP ($p<0.001$). We also assessed the significance of two NF-κB2 genetic polymorphisms, rs12769316 and rs7897947. Both polymorphisms were associated with lymph node infiltration ($p=0.045$ and $p=0.009$, respectively). In addition, rs12769316 AA homozygotes relapsed less often compared to G allele carriers ($p=0.029$). Moreover, rs7897947 allele frequencies differed significantly between CRC patients and healthy controls ($p<0.001$) and the minor allele (G) was associated with reduced risk for developing CRC ($p<0.001$, OR: 0.527, 95% CI: 0.387–0.717). In conclusion, the alternative NF-κB pathway appears deregulated in CRC. Moreover, NF-κB2 and RELB expression levels seem to be significant for the clinical outcome of CRC patients and rs7897947 appears to be a risk factor for CRC development.

Introduction

Colorectal cancer (CRC) is the third most common type of malignancy in men and the second in women and represents the third most common cause of death from cancer in both men and women and the seventh overall cause of death in Europe. Moreover, it is predicted to rise in frequency by 20% by the year 2030 [1]. CRC most commonly arises from adenomatous polyps frequently accompanied by an inflammatory response. A major pathway involved in inflammation is the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling cascade. NF-κB is a pleiotropic transcription factor mediating transcription of cytokines and chemokines that are important in immune responses and inflammation as well as in cancer development, progression and resistance to treatment [2,3]. NF-κB signalling occurs through two major interconnected pathways: the canonical or classical and the non-canonical or alternative NF-κB pathway and is comprised of five members; NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-rel [4]. The classical pathway has been extensively studied but the alternative pathway has only recently attracted attention in cancer and remains largely underexplored in CRC. In the alternative pathway, stimulation of the TNF-α receptor family members, namely lymphotoxin β receptor (LT-βR), B-cell activating factor (BAFFR), receptor activator of NF-κB (RANK), CD40 and other surface receptors leads to activation

Abbreviations: NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; CRC, colorectal cancer, NSCLC, non-small cell lung cancer; SNP, Single nucleotide polymorphism; PCR, Polymerase chain reaction; qRT-PCR, real time quantitative reverse transcription PCR; NN, Non-neoplastic; TTP, Time to progression; OS, Overall survival; FFPE, Formalin-fixed paraffin-embedded; LN, Lymph node; HWE, Hardy Weinberg equilibrium; TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression.

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of NF-κB-inducing kinase (NIK), which subsequently leads to proteolytic cleavage of NF-κB2 (p100) to NFKB2 (p52). NFKB2 exerts it transactivating function through the formation of an heterodimer with RELB [5]. RELB and NF-κB2 are the most studied members and form the most prominent dimer of the alternative NF-κB pathway, probably because of their binding stability [6].

We have previously shown the deregulation of these two molecules in non-small cell lung cancer (NSCLC) [7,8] where their levels were elevated compared to normal tissue. Similarly, increased levels of NF-κB2 and RELB and activation of the alternative NF-κB pathway have been demonstrated in breast and prostate tumours and in numerous cancer cell lines [2,9,10]. Regarding CRC, there is scarce information on their role in the pathogenesis and progression of the disease mainly from a few recently published studies. In preclinical studies, *NFKB2*$^{-/-}$ mice did not develop adenomas and exhibited elevated epithelial cell apoptosis compared to wild type mice following the induction of chronic colitis using dextran sodium sulfate and the DNA damaging agent azoxymethane (AOM/DSS) [11]. Additionally, activation of RELB in four specimens of colon cancer and in the colon cancer cell line HT29 has been suggested [12]. Furthermore, RelB silencing increased growth of the colon cancer cell line DLD-1, increased the cytotoxic effect of the chemotherapeutic agent 5-FU and impaired migration and invasion potential of the cells revealing an oncogenic role in these cells [13]. Elevated RELB and NFKB2 protein levels were recently reported in colon cancer specimens in tissue microarrays compared to adjacent non-neoplastic (NN) tissues and they were indicative of prognosis [13,14].

Although there are these very few reports on protein levels of RELB and NF-κB2 in CRC, there is lack of information on gene expression levels and on the role of single nucleotide polymorphisms (SNPs) of the *NFKB2* gene in these tumours. We have previously shown an elevated RELB gene expression in tumour compared to normal tissue in NSCLC and an association of *NFKB2* SNPs rs12769316 and rs7897947 with susceptibility to NSCLC, patient survival and response to therapy [8,15]. Rs12769316 is located on the promoter of the *NFKB2* gene within a binding site for a CCCTC-binding transcription factor (CTCF) and has been associated with NFKB2 mRNA and protein levels in NSCLC [15]. Rs7897947 is an intronic SNP of the *NFKB2* gene. It lies within intron 8 which is retained by a non-coding RNA transcript, but its function remains unknown.

The aim of this study was to evaluate the clinical significance of NF-κB2 and RELB in CRC at a multiple level. We have assessed their gene and protein expression in CRC tissues to evaluate their prognostic value. Moreover, we have evaluated, for the first time, the protein expression of these two proteins in stromal cells, given the importance of stroma-tumour interactions. In addition, we have genotyped CRC patients and healthy individuals to assess the value of the two *NFKB2* SNPs, rs7897947 and rs12769316, in prognosis and in susceptibility to developing CRC.

Materials and methods

Tissue and blood specimens

The study was approved by the Committee on Research and Ethics and the Scientific Committee of the University Hospital of Patras, Greece (20691/24-10-16). The study comprised of formalin-fixed paraffin-embedded (FFPE) tumour and non-neoplastic (NN) tissue samples from 92 patients with CRC. Blood samples were also provided by 291 patients with CRC (raising the total number of CRC patients to 383) and 300 healthy donors together with informed consent. All cases and controls were genetically unrelated Greek individuals. The tissue samples were retrieved from the archives of the Department of Pathology (University Hospital of Patras) and the blood samples were either prospectively collected or retrieved from the archives of the Clinical and Molecular Oncology Laboratory of the University of Patras.

Table 1
Clinicopathological characteristics and survival data of CRC patients. Abbreviations: NA, data not available or unknown.

Clinicopathological characteristics	*Cases n* (%)
Total	383
Age (years) Median (range)	67 (28–90)
Sex	
Male	232 (60.6)
Female	151 (39.4)
Primary location	
Ascending colon	105 (27.4)
Descending colon	167 (43.6)
Rectum	94 (24.5)
NA	17 (4.4)
Stage	
I	12 (3.1)
II	108 (28.2)
III	112 (29.2)
IV	129 (33.7)
NA	22 (5.7)
Grade	
I	40 (10.4)
II	249 (65.0)
III	58 (15.1)
NA	36 (9.4)
Lymph node infiltration	121 (31.6)
No	242 (63.2)
Yes	20 (5.2)
NA	
Relapse	
No	99 (25.8)
Yes	192 (50.1)
NA	92 (24.0)
Survival (overall)	
Dead	137 (35.8)
Alive	167 (43.6)
NA	79 (20.6)
Survival (5 years)	
Dead	130 (33.9)
Alive	174 (45.4)
NA	79 (20.6)

Overall survival (OS) was assessed after a follow up period of 125 months using past medical histories from the archives of the Division of Oncology or through direct personal contact (via phone or in person). Clinicopathological characteristics of CRC patients are listed in Table 1.

Immunohistochemistry and evaluation of protein expression

Immunohistochemical analysis was performed using FFPE tissue sections from 92 patients with CRC as described previously [7]. Tumour, NN tissue adjacent to the tumour and distant NN tissue were assessed. In each location epithelial and stromal cells were separately assessed. A mouse monoclonal primary antibody (sc-7386, Santa Cruz) was used for the detection of NF-κB2 and a rabbit polyclonal antibody was used against RELB (sc-226, Santa Cruz). The Envision detection kit (DAKO) was used for signal detection and diaminobenzidine was used as the chromogen according to the manufacturer's instructions. Protein blocking solution was used instead of the primary antibodies in consecutive sections to ensure specificity. Inflammatory cells of the tumour microenvironment were used as internal positive controls.

Evaluation of the immunohistochemical staining was performed as described previously [16]. An experienced pathologist (VT) scored blindly each slide. Representative areas were selected at low (× 100) magnification and cells were counted at a 400 × magnification. At least 1000 cells were counted in each section. Cytoplasmic, membrane and nuclear staining were evaluated separately for each marker as the percentage of cells with positive staining (0–100 in 10-point increments). The intensity of staining was evaluated using a three-tiered scale. A to-

tal score was calculated by multiplying the percentage of positive cells by the intensity of staining (range 0–300). Microphotographs were obtained by Lumenera's INFINITY HD digital camera (Lumenera Co, OTT, Canada) mounted on an Olympus BX41 microscope (Olympus Europa SE & Co., Hamburg, Germany).

Gene expression analysis

Tumour (92 samples) and normal (61 samples) FFPE tissue sections from 92 CRC patients were used to assess *NFKB2* and *RELB* gene expression. Total RNA was extracted from tissues using the NucleoSpin Total RNA FFPE Kit (Macherey-Nagel, GmbH & Co, Düren, Germany) and reverse transcribed using Superscript III Reverse Transcriptase (Life Technologies) and random nonamers as described previously [17]. Expression of *NFKB2* and *RELB* was quantified using real time quantitative polymerase chain reaction (qRT-PCR) assays as described previously [8]. Expression levels were normalised to Alu-Sq levels that were found to be stably expressed in normal and neoplastic tissue of different grades and stages [17].

Genotyping

CRC patients (383) and healthy donors (300) were genotyped for two *NFKB2* SNPs, rs7897947 and rs12769316 as previously described [15]. Briefly, DNA was isolated from normal FFPE tissue sections or blood samples using the QIAamp DNA FFPE Tissue kit (Qiagen Ltd., Crawley, UK) and QIAamp mini blood kit (QIAGEN), respectively. Genotyping was performed using real time PCR with SYBR Green I and melting curve analysis [18]. All reactions were performed in duplicate. Reactions without template were included as a control for contamination. The genotype of several samples was confirmed by sequencing as previously described at CEMIA SA (University of Thessaly, Greece) [15].

Statistical analysis

Statistical analysis was performed with the IBM SPSS Statistics version 25 software (IBM corp., Armonk, NY, USA). Possible relations between variables were assessed with Spearman's correlation. Associations between protein or gene expression and clinicopathological parameters or genotypes were assessed with the Kruskal–Wallis or the Mann–Whitney tests or the χ^2 test. Survival rates were assessed using the Kaplan–Meier method and compared with the log rank test. Meaningful cut off points for protein and gene expression were set using the bioinformatics tool for biomarker assessment and outcome-based optimisation, X-tile, as opposed to arbitrary cut off points by percentiles [19]. Consistency of the genotype frequencies to Hardy-Weinberg expectations was assessed with the χ^2 test. Logistic regression and odds ratios were used to evaluate associations between genotypes and CRC susceptibility. Statistical significance was defined as $p<0.05$ for all comparisons.

Results

Subcellular localisation of NF-κB2 and RELB protein immunostaining

NF-κB2 protein was expressed in the cytoplasm of the tumour cells in all cases (Table 2) (Fig. 1). Focal membrane staining was also seen in a minority of cases (23/92). Nuclear staining was seen in a few cases, but it was so faint and rare that it was not further analysed. Epithelial cells of adjacent and distant non neoplastic (NN) mucosa displayed only cytoplasmic expression, with membranous staining seen in one case of adjacent NN cells. NF-κB2 protein was also expressed in the cytoplasm of stromal and endothelial cells in all cases analysed (both cancerous and NN tissue).

RELB protein was expressed in the cytoplasm and the nucleus of epithelial cells in both carcinomas and NN mucosa (adjacent and distant) (Fig. 2). Moreover, RELB protein was observed in the nucleus (at lower levels) and in the cytoplasm of stromal and endothelial cells (Fig. 2).

Cytoplasmic protein expression differed between tumour and normal cells and their respective stromal cells

Cytoplasmic NF-κB2 protein levels were similar between tumour cells and adjacent NN cells ($p=0.173$), but they were significantly higher in tumour cells compared to distant NN cells ($p<0.001$) (Fig. 3A). Additionally, expression combined with intensity of staining was stronger in tumour cells compared to distant NN cells ($p< 0.001$). Cancer-associated stromal cells displayed higher NF-κB2 protein levels compared to stromal cells surrounding either adjacent NN cells ($p<0.001$) or distant NN cells ($p<0.001$) (Fig. 3B).

Cytoplasmic RELB protein expression was higher in tumour cells compared to adjacent ($p=0.009$) and distant ($p<0.001$) NN cells (Fig. 3A). Nuclear RELB levels, however, did not differ significantly between tumour and NN cells. Nevertheless, nuclear and cytoplasmic RELB levels in the tumour were positively correlated ($r=0.547$, $p<0.001$). Regarding stromal cells, RELB levels were higher in cancer-associated stromal cells compared to stromal cells in NN samples (both in adjacent, $p<0.001$, and distant, $p<0.001$, NN samples) (Fig. 3B). Furthermore, a moderate positive correlation was noted between cytoplasmic RELB and cytoplasmic NF-κB2 levels in tumour cells ($r=0.399$, $p<0.001$).

Relationship of protein expression with clinicopathological variables

Cytoplasmic NF-κB2 expression was positively correlated with the grade of the tumour ($p=0.026$) with higher grade tumours expressing higher levels of NF-κB2 (Fig. 3C). Moreover, cytoplasmic NF-κB2 expression differed according to the anatomical site ($p=0.032$). Levels were higher in tumours of the rectum compared to tumours in the colon, while left side tumours had the lowest cytoplasmic NF-κB2 levels (Fig. 3D). NF-κB2 expression was independent of stage, lymph node (LN) infiltration status and relapse. Regarding RelB expression in the tumour, it was independent of stage, grade, primary site, LN status and relapse status.

RELB gene expression differs between tumour and normal tissue

NFKB2 gene expression was similar between tumour and NN tissue ($p=0.110$). On the contrary, *RELB* gene expression was lower in tumour tissue compared to NN tissue ($p=0.003$) when samples were analysed in a pairwise manner (tumour and matching NN tissue). Moreover, *RELB* and *NFKB2* gene expression levels were strongly related in NN ($r=0.939$, $p< 0.001$) and in cancer tissue ($r=0.958$, $p<0.001$).

RELB protein (cytoplasmic and nuclear) expression was weakly correlated with gene expression levels in tumour tissue ($r=0.343$, $p=0.002$) but not in normal tissue ($p=0.460$). On the contrary, *NFKB2* gene expression was not associated with NF-κB2 protein levels.

NFKB2 and *RELB* gene expression at the tumour was unrelated to stage ($p=0.686$ and $p=0.445$), grade ($p=0.325$ and 0.390), primary site ($p=0.254$ and $p=0.306$), LN status ($p=0.521$ and $p=0.569$) and relapse status ($p=0.192$ and $p=0.272$).

Prognostic value of gene and protein expression

To assess prognostic value of protein or mRNA levels, the most meaningful cut off point was used in each case. High cytoplasmic (above 90) and nuclear (above 80) RELB expression in tumour cells was associated with improved OS ($p=0.032$ and $p=0.040$, respectively; Fig. 4A and B). Regarding stromal expression, high overall RELB expression (above 60) was associated with improved OS ($p=0.028$; Fig. 4C) and improved 5-year survival ($p=0.025$). TTP was independent of NF-κB2 and RELB protein expression in the tumour.

Regarding tumour mRNA levels of the two genes studied, a prognostic value was observed for 5-year and OS. Notably, higher relative

Table 2
NF-κB2 and RELB mean protein expression (± standard deviation) in the cytoplasm (C), membrane (M) or nucleus (N) of tumour cells, adjacent non-neoplastic (adj NN), distant non-neoplastic tissue (NN) and associated stroma. * single case.

NF-κB2 mean protein expression (±SD)								
Tumour Epithelial cells			Adj NN Epithelial cells			Distant NN Epithelial cells		
C	M	Stroma	C	M	Stroma	C	M	Stroma
86.3 ± 20.9	1.9 ± 4.5	82.0 ± 18.4	83.3 ± 14.3	5*	39.5 ± 23.9	65.9 ± 22.7	0	27.2 ± 15.3
RELB mean protein expression (±SD)								
Tumour Epithelial cells			Adj NN Epithelial cells			Distant NN Epithelial cells		
C	N	Stroma	C	N	Stroma	C	N	Stroma
87.3 ± 20.9	79.1 ± 15.2	64.8 ± 18.2	57.1 ± 25.9	75.2 ± 9.3	25.2 ± 12.5	42.2 ± 26.1	77.8 ± 10.4	33.7 ± 16.4

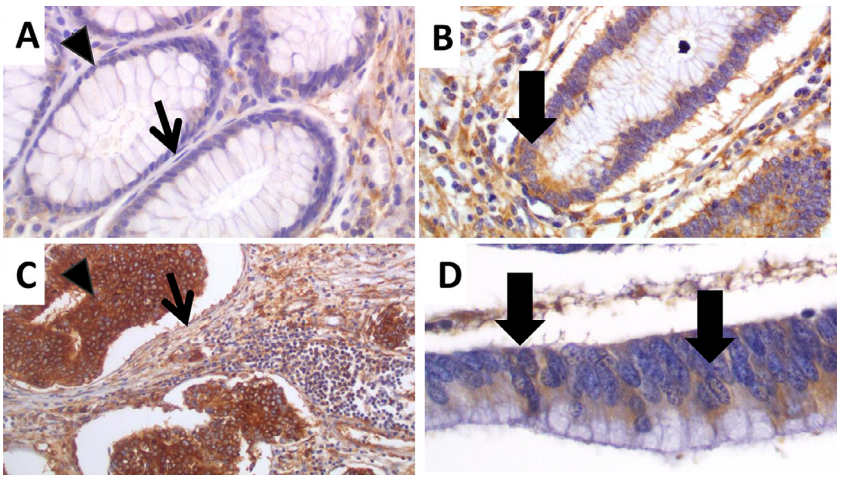

Fig. 1. NF-κB2 expression in non-neoplastic and cancerous colon. A. Expression in distal non neoplastic mucosa is low (original magnification X40). B. Expression in adjacent non neoplastic mucosa with rare membranous (thick arrow) staining (original magnification X40). C. High cytoplasmic expression in tumour and cancer-associated stromal cells (original magnification X20) D. Faint, rare nuclear expression, barely recognised in the epithelial cells (thick arrow) (original magnification X40) (arrow: stromal cell, arrowhead: epithelial cells).

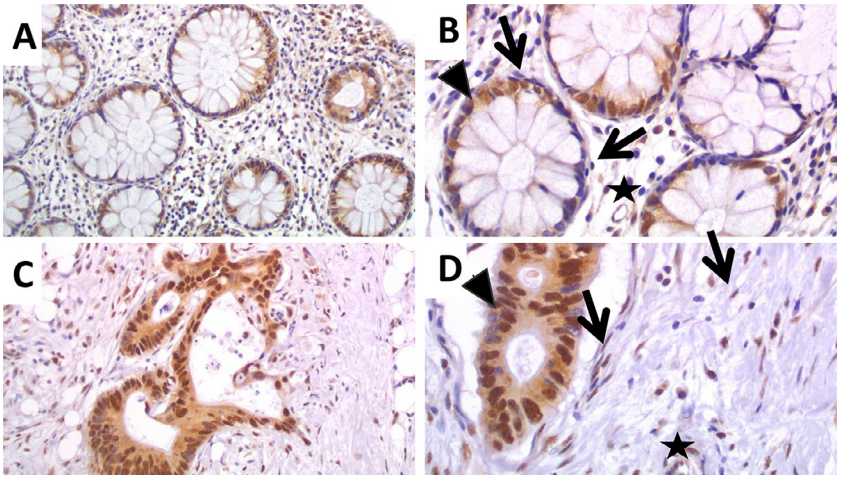

Fig. 2. RELB expression in non-neoplastic and neoplastic colon. Expression is lower in non-neoplastic (A, original magnification X20, B, original magnification X40) epithelial (arrowhead) and stromal cells (arrow) compared to the tumour (C, original magnification X20, D, original magnification X40). Note expression in endothelial cells (star).

NFKB2 gene expression (above 2.51) was related to longer survival ($p = 0.018$ and $p = 0.038$, respectively; Fig. 4D) while *RELB* mRNA levels were independent of survival ($p = 0.396$). High *RELB* mRNA levels (above 3.32) were, however, related to increased TTP ($p = 0.025$; Fig. 4E). Similarly, high *NFKB2* mRNA levels (above 2.51) were associated with longer TTP ($p<0.001$; Fig. 4F).

Frequencies of genotypes and alleles across subpopulations-association of NFKB2 rs7897947 with risk of developing CRC

Three hundred healthy donors and 383 CRC patients were successfully genotyped for rs7897947 and rs12769316 SNPs (Table 3). Most individuals were homozygous T for rs7897947 and homozygous G for rs12769316 (Table 3). In healthy individuals, rs7897947 T frequency was lower than the frequency reported for the European population (0.683 *vs* 0.796) while frequencies of the rs12769316 G allele in this study and the European population were similar (0.775 vs 0.814) (National Center for Biotechnology Information, NCBI, https://www.ncbi.nlm.nih.gov/snp/). Rs7897947 was in Hardy Weinberg equilibrium (HWE) in healthy individuals and in CRC patients, while rs12769316 SNP was not, meaning that any statistical associations with disease that arise in the study should be corrected to avoid bias [20]. Genotyping error is unlikely to have occurred as assured by the agreement of genotypes observed through two different genotyping methods and by the lack of heterozygosity gain seen in the genotypes, suggesting a natural cause of departure from HWE [21].

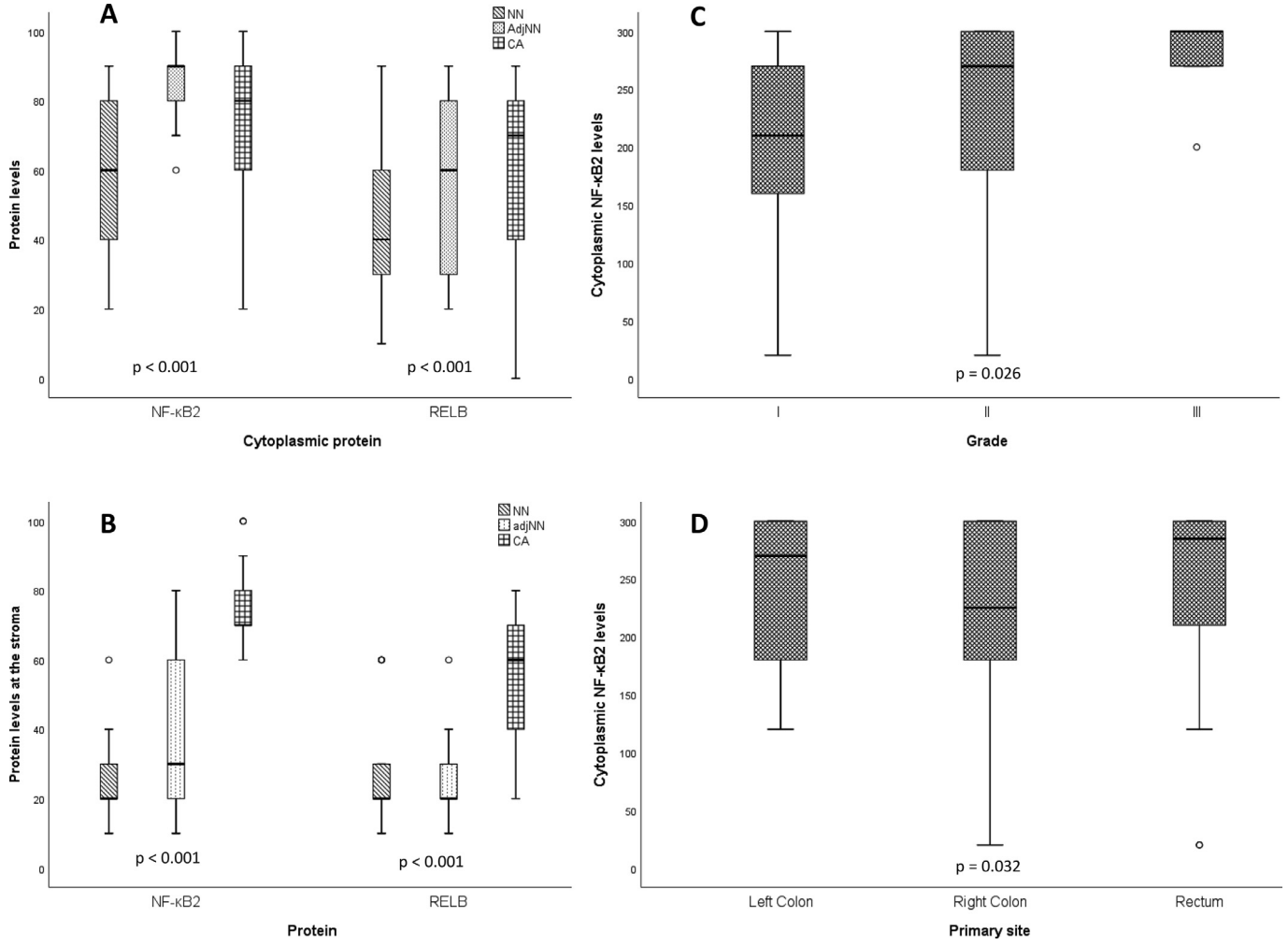

Fig. 3. A. Boxplots to illustrate cytoplasmic NF- κB2 and RELB levels in tumour tissue (CA), adjacent non-neoplastic tissue (adjNN) and distant NN. **B.** Box plots illustrating the difference in expression levels of proteins NF-κB2 and RELB at the stroma surrounding the tumour (CA), the tumour-adjacent non-neoplastic tissue (adjNN) and distant NN. **C.** Boxplots showing the difference in cytoplasmic NF-κB2 protein levels in relation to the grade of the tumour. **D.** Boxplots illustrating the difference in cytoplasmic NF-κB2 expression in tumours from different anatomical sites.

Table 3
Genotype distribution of rs7897947 and rs12769316 in cases (CRC patients) and controls (healthy individuals).

SNP genotype	Counts controls *N* (%)	Cases *N* (%)	χ^2 *P*	Univariate analysis *P*	OR (95% CI)
Total	300	383			
rs7897947					
TT	145 (48.33)	245 (63.97)	<0.001	<0.001	0.588 (0.463–0.747)
TG	120 (40.67)	117 (30.54)			
GG	35 (11.67)	21 (6.27)			
TT vs TG & GG	145 (48.33)	245 (63.97)	<0.001	<0.001	0.527 (0.387–0.717)
	155 (51.67)	138 (36.03)			
T allele frequency	*0.683*	*0.792*	<0.001		
G allele frequency	*0.317*	*0.208*			
Hardy Weinberg P	0.189	0.162			
rs12769316					
GG	195 (65)	254 (66.32)	0.937	–	1
GA	75 (25)	92 (24.02)		0.836	1.056 (0.630–1.770)
AA	30 (10)	37 (9.66)		0.985	0.995 (0.562–1.759)
GG vs GA & AA	195 (65)	254 (66.32)	0.719	0.719	0.943 (0.686–1.296)
	105 (35)	129 (33.68)			
G allele frequency	*0.775*	*0.783*	*0.706*		
A allele frequency	*0.225*	*0.217*			
Hardy Weinberg *P*	<0.001	<0.001			

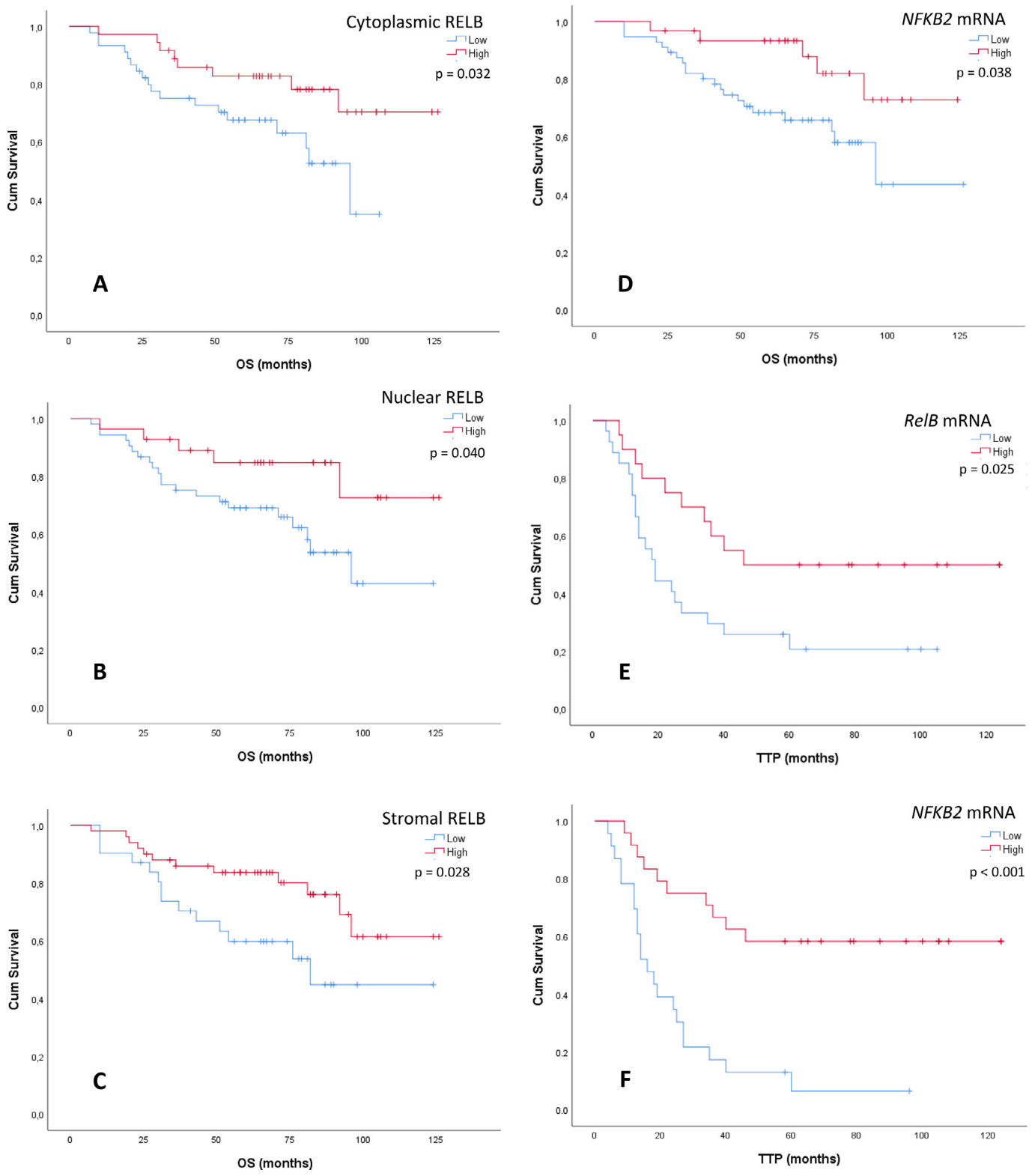

Fig. 4. Kaplan Meier plots illustrating the difference in survival depending on RELB protein levels in the tumour (A) cytoplasm, (B) nucleus and (C) stroma; the difference in survival depending on *NFKB2* mRNA levels (D); the difference in TTP depending on *RELB* (E) or *NFKB2* mRNA levels (F).

Rs12769316 allele frequencies were similar between healthy individuals and CRC patients ($p = 0.706$) whereas rs8997947 allele frequencies differed significantly ($p<0.001$) (Table 3). Notably, there was a lower frequency of the minor allele (rs8997947 G) and a higher frequency of TT homozygotes in CRC patients compared to healthy controls ($p<0.001$ for both). Accordingly, the presence of the G allele was associated with reduced risk for developing CRC by 52.7% (Table 3).

Association of the NFKB2 SNPs with clinicopathological parameters and gene and protein expression levels

Rs7897947 was associated with the primary site. Patients with tumours of the right colon harboured more frequently the G allele (Table 4). Both SNPs correlated with stage and lymph node infiltration. The presence of the minor allele was associated with a higher stage

Table 4
Genotypes of rs7897947 and rs12769316 in relation to clinicopathological parameters. Abbreviations: LN, Lymph Nodes; RC, right (ascending) colon; LC, left (descending) colon; R, rectum.

SNP Genotype	Counts (%) Grade			Stage				Primary site			LN infiltration		Relapse	
rs7897947	**I**	**II**	**III**	**I**	**II**	**III**	**IV**	**RC**	**LC**	**R**	**No**	**Yes**	**No**	**Yes**
Total	40	249	58	12	108	112	129	105	167	94	121	242	99	192
TT	27 (67.5)	160 (64.3)	32 (55.2)	9 (75)	78 (72.2)	70 (62.5)	71 (55.0)	57 (54.3)	113 (67.7)	60 (63.8)	88 (72.7)	142 (58.7)	61 (61.6)	123 (64.1)
TG	10 (25)	74 (29.7)	24 (41.4)	3 (25)	24 (22.2)	34 (30.4)	51 (39.5)	44 (41.9)	40 (23.9)	31 (32.9)	27 (22.3)	85 (35.1)	28 (28.3)	60 (31.2)
GG	3 (7.5)	15 (6.0)	2 (3.4)	0	6 (5.6)	8 (7.1)	7 (5.4)	4 (3.8)	14 (8.4)	3 (3.2)	6 (4.9)	15 (6.2)	10 (0.1)	9 (4.7)
P	0.389			0.126				**0.015**			**0.030**		0.204	
TT vs TG&GG	27 (67.5) vs 13 (32.5)	160 (64.3) vs 89 (35.7)	32 (55.2) vs 26 (44.8)	9 (75) vs 3 (25)	78 (72.2) vs 30 (27.8)	70 (62.5) vs 42 (37.5)	71 (55.0) vs (45.0)	57 (54.3) vs 48 (45.7)	113 (67.7) vs 54 (32.3)	60 (63.8) vs 34 (36.2)	88 (72.7) vs 33 (27.3)	142 (58.7) vs 100 (41.3)	61 (61.6) vs 38 (38.4)	123 (64.1) vs 69 (35.9)
P	0.360			**0.042**				0.082			**0.009**		0.682	
	I	**II**	**III**	**I**	**II**	**III**	**IV**	**RC**	**LC**	**R**	**No**	**Yes**	**No**	**Yes**
rs12769316														
Total	40	249	58	12	109	111	129	105	167	94	122	241	99	193
GG	22 (55.0)	170 (68.3)	42 (72.4)	7 (58.3)	65 (59.6)	67 (60.4)	101 (78.3)	64 (60.9)	114 (68.3)	66 (70.2)	72 (59.0)	169 (70.1)	66 (66.7)	145 (75.1)
AG	13 (32.5)	55 (22.1)	15 (25.9)	4 (0.3)	31 (28.4)	30 (27.0)	22 (17.1)	28 (26.7)	38 (22.7)	22 (23.4)	36 (29.5)	52 (21.6)	19 (19.2)	38 (19.7)
AA	5 (12.5)	24 (9.6)	1 (1.72)	1 (8.3)	13 (11.9)	14 (12.6)	6 (4.6)	13 (12.4)	15 (8.9)	6 (6.4)	14 (11.5)	20 (8.3)	14 (14.1)	10 (6.2)
P	0.135			**0.034**				0.535			0.106		**0.029**	
GG vs AG&AA	22 (55.0) vs 18 (45.0)	170 (68.3) vs 79 (31.7)	42 (72.4) vs 16 (27.6)	7 (58.3) vs 5 (41.7)	65 (59.6) vs 44 (40.4)	67 (60.4) vs 44 (39.6)	101 (78.3) vs 28 (21.7)	64 (60.9) vs 41 (39.1)	114 (68.3) vs 53 (31.7)	66 (70.2) vs 28 (29.8)	72 (59.0) vs 50 (41.0)	169 (70.1) vs 72 (29.9)	66 (66.7) vs 33 (33.3)	145 (75.1) vs 48 (24.9)
P	0.169			**0.003**				0.322			**0.045**		0.131	

for the rs7897947 SNP (G) and a lower stage for the rs12769316 (A) (Table 4). Accordingly, the rs7897947 G allele was found more frequently in patients with positive lymph nodes compared to patients without lymph node infiltration, while the rs12769316 A allele was less frequent (Table 4). Rs12769316 was also correlated with relapse status with the AA homozygotes being less frequent amongst patients who relapsed (Table 4). Both rs7897947 and rs12769316 were unrelated to overall survival ($p = 0.167$ and $p = 0.454$, respectively) and time to progression ($p = 0.512$ and $p = 0.751$, respectively).

Both *NFKB2* SNPs studied were unrelated to *NFKB2* gene expression but rs12769316 was related to membrane levels of NF-κB2 protein in tumour tissue and rs7897947 was related to total (cytoplasmic and membrane) tumour NF-κB2 protein levels. Notably, the rs12769316 A allele was associated with higher levels of membranous NF-κB2 ($p = 0.023$ for the recessive allele model and $p = 0.048$ when all three genotypes were analysed). Moreover, the rs7897947 TT genotype was associated with lower NF-κB2 expression ($p = 0.033$).

Discussion

The importance of the alternative NF-κB pathway is being increasingly recognised in cancer due to its involvement in various aspects of oncogenesis and disease progression including immune system homoeostasis [2,3,22]. Although the role of the classical NF-κB pathway has been extensively studied in CRC, the role of the alternative pathway remains underexplored. We have previously shown the deregulation of this pathway in NSCLC [7,8]. In this context, we assessed gene and protein expression of NF-κB2 and RELB in tumour and non-neoplastic tissues as well as in stromal cells whose importance in tumour development is being increasingly recognised. Additionally, two SNPs of the *NFKB2* gene have been included in our analysis regarding the clinical significance of NF-κB2 and RELB in CRC.

RELB protein was observed mainly in the cytoplasm and less in the nucleus of tumour and normal cells. Cytoplasmic levels were significantly higher in tumour tissue compared to normal tissue, while there was no significant difference in nuclear RELB levels. Our results are in agreement with a previous report with regard to the cytoplasmic expression of RELB assessed in CRC tissue microarrays [14]. However, a significant difference in nuclear RELB levels between tumour and normal tissue has been previously reported in tissue microarrays with tumour and adjacent NN samples from Chinese patients [14]. These differences may reflect differences in the population studied and the methodology used (i.e. TMA vs whole section analysis). RELB protein is regulated by post-translational modifications perhaps playing a role in the accumulation of RELB in the cytoplasm of tumour cells and in the tumour-associated stroma compared to NN cells [23]. RELB can also form dimers with the p50 subunit of NF-κB1 (classical pathway) and is also regulated transcriptionally by enhancer of zeste homologue 2 (EZH2) in combination with RELA, another member of the classical pathway, adding to the complexity of RELB regulation and its role in cancer [24,25]. The interconnection between the classical and the alternative NF-κB pathway is further illustrated by the induction of *RELB* transcription by RELA. Moreover, RELB sequesters RELA in the cytoplasm upon stimulation of the classical NF-κB pathway, lowering the strong inflammatory response of the classical pathway [26,27]. Besides, the induction of the target genes varies not only depending on the stimulus but also on the cell type [2] which could explain the discrepancies in protein expression and localisation observed in CRC compared to NSCLC [7,8].

RELB gene expression was lower in tumour compared to NN tissue. Analysis of TCGA (The Cancer Genome Atlas) and GTEx (Genotype-Tissue Expression) gene expression data from microarrays revealed a lack of a statistically significant difference between tumour and non-malignant tissue in *RELB* gene expression, in contrast to our findings (Suppl Fig. 1A) [28]. However, when TCGA data were analysed separately, lower levels were observed in the tumour compared to NN tissues, although without reaching statistical significance (Suppl. Fig. 1B). This discrepancy may be due to the smaller sample size of our study. However, it should also be noted that qPCR analysis using the appropriate reference gene is a more accurate technique compared to expression microarrays. Thus, a difference or lack of in *RELB* gene expression between tumour and NN tissue cannot be stated with confidence until a larger number of samples are analysed by qRT-PCR.

With regard to NF-κB2, cytoplasmic expression was higher in neoplastic tissue compared to normal tissue samples, in agreement with the only other report to our knowledge of the expression of NF-κB2 in CRC tissues [14] where cytoplasmic and nuclear immunostaining was observed. In our study, a low level of membrane staining was observed in tumour tissues but no discrete nuclear staining was noted. This finding is in agreement with the Protein Atlas NF-κB2 protein localisation in CRC (https://www.proteinatlas.org/ENSG00000077150-NFKB2/pathology/colorectal+cancer#imid_2581630) whereby immunostaining of CRC tissues with three different antibodies revealed cytoplasmic and membrane staining only [29]. *NFKB2* gene expression did not follow the patterns of protein expression, supported by the observed lack of correlation between protein and gene expression levels. mRNA levels were similar in tumour and normal tissue in accordance with the known post transcriptional regulation mechanism of NF-κB2 in addition to the self-regulatory transcriptional regulation [30–32]. In further support, *NFKB2* mRNA levels assessed by microarrays and publicly available in TCGA and GTEx databases did not differ between colon adenocarcinoma and normal tissue samples (Supplementary Fig. 1C).

In addition to neoplastic cells, we assessed, for the first time, the levels of the two proteins in the stroma. Of interest are the significantly higher levels observed for both RELB and NF-κB2 proteins in the tumour-associated stroma compared to the non-neoplastic stroma. It is known that the stroma surrounding the tumour is “activated” by the tumour cells [33]. Along these lines, here, we present evidence that amongst other factors, the alternative pathway of NF-κB is also deregulated in the tumour stroma. In further support, a proto-oncogenic role of RelB in the tumour microenvironment has been demonstrated in a model of T acute lymphoblastic leukaemia [34].

The presence of cytoplasmic NF-κB2 expression and not nuclear may suggest that there are higher levels of p100 compared to p52 in the tumour. In this case, higher levels of NF-κB2 would indeed correlate with improved OS or TTP since p100 and not p52 has been shown to inhibit anchorage-independent growth [35]. Although an association of protein NF-κB2 levels and survival was not identified, an improved survival was noted in patients with higher gene expression of *NFKB2* which is in line with the survival benefit revealed by analysing publicly available gene expression data from 1003 colon adenocarcinoma patients (Suppl. Fig. 2A) [28]. Moreover, these findings are also in line with the higher cytoplasmic NF-κB2 levels observed in rectal cancer patients compared to colon cancer patients, combined with the survival benefit noted in patients whose primary site was the rectum compared to patients with tumours in the colon [36].

Higher levels of cytoplasmic and nuclear RELB protein in the tumour and in the stroma were associated with improved survival. In addition, high *NFKB2* and *RELB* mRNA levels correlated with longer TTP. Publicly available data on gene expression are in agreement with our findings as higher levels of *NFKB2* or *RELB* gene expression in 1003 patients with colon adenocarcinoma were associated with improved survival (Suppl. Fig. 2B) [37]. However, in contrast to our and the publicly available data we present, other studies have shown that high RELB expression is associated with poor prognosis [14]. Number of patients studied, patient population and methodology used may account for the discrepancies. In addition, an opposing role for RELB may exist, comprising both a tumour promoting and an anti-oncogenic role in a cellular and stage–specific pattern, as has been seen for other markers in the NF-κB alternative pathway or elsewhere. For example, elevated levels of the inhibitor of kappa-B (IκB) protein like B-cell lymphoma 3-encoded protein (BCL3) were found to be associated with poor OS in stages I-II and

to improved OS in stages III-IV patients with NSCLC [8]. Moreover, the role of NF-κB has been conflicting in certain cases, where inhibition of NF-κB promoted tumorigenesis [38]. In addition, the complexity of NF-κB pathway and the interplay between classical and alternative mode of activation should be considered when analysing their components. Further study of the role of RELB at protein and mRNA levels is needed to elucidate this matter.

Gene expression was unrelated to both *NFKB2* SNPs studied. Rs12769316 A allele appeared to be related to membrane NF-κB2 levels and rs7897947 was related to total NF-κB2 in tumour samples. We have previously shown that rs7897947 was unrelated to protein and gene expression in NSCLC and that rs12769316 was related to both mRNA and protein levels in NSCLC [15]. This is not surprising considering the location of rs12769316 within a transcription factor binding site on the gene promoter. It is possible that there is a different transcription activation mechanism in CRC compared to NSCLC which is supported by the presence of other transcription factor binding sites in the promoter as suggested by the PROMO software [39].

Furthermore, despite a correlation noted between both SNPs and stage and lymph node infiltration, OS and TTP were independent of these two SNPs although the rs12769316 AA genotype was more frequent amongst patients who remained disease-free. The role and mechanisms of action through which these SNPs may exert an effect remain unknown and there is scarce information about their relationship with cancer. Of interest is the influence of rs7897947 on sleep disturbance, trait anxiety, and poorer social well-being of oncology patients and their family caregivers [40]. Moreover, rs12769316 has been related to response rate of multiple myeloma patients treated with bortezomib-based regimens, which is a proteasome inhibitor and suppresses NF-κB activation [41]. Taken together, these findings along with our observations, indicate that these polymorphisms have a role in tumour progression and response to therapy. However, further studies are needed to unravel their mechanistic input and potential therapeutic targeting.

In addition to its role in cancer progression, rs7897947 was found to be a risk factor for developing CRC with carriers of the G allele displaying a lower susceptibility to CRC compared to TT carriers. Similarly, TT individuals are at greater risk for developing NSCLC [15] while rs7897947 genotype does not affect risk of multiple myeloma development [41] suggesting a possible organ specific effect of this SNP.

To conclude, our findings suggest that the alternative NF-κB2 pathway is deregulated in CRC not only at the tumour but also at the associated stroma and these changes could be useful in determining patients' prognosis. The most important differences were noted at the protein level, possibly before activation of the alternative pathway as significant differences were seen in cytoplasmic protein levels. NF-κB activation is achieved by various stimuli but this activation cannot be strictly divided into two different pathways (non-canonical and canonical). There are various points that the two pathways interconnect and regulate each other adding to the complexity of the regulation of the NF-κB signalling that remains to be clarified. Additionally, the role of SNPs in this pathway should also be considered as we observed an association of rs12769316 with susceptibility to CRC.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2020.100912.

CRediT authorship contribution statement

Anna Antonacopoulou: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Anastasia E. Kottorou:** Methodology, Investigation, Writing - review & editing. **Foteinos-Ioannis Dimitrakopoulos:** Conceptualization, Investigation, Writing - review & editing. **Stella Marousi:** Resources. **Foteini Kalofonou:** Writing - review & editing. **Angelos Koutras:** Resources, Writing - review & editing. **Thomas Makatsoris:** Resources, Writing - review & editing. **Vassiliki Tzelepi:** Investigation, Writing - review & editing. **Haralabos P. Kalofonos:** Project administration, Resources, Writing - review & editing.

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