Synergism between K-ras^{Val12} and mutant *Apc* accelerates murine large intestinal tumourigenesis

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Abstract. K-*ras* (*KRAS*) is mutated in 40-50% of human colorectal adenomas and carcinomas and plays key roles in cell proliferation, apoptosis, motility and differentiation, but its functional contribution to intestinal tumourigenesis *in vivo* remains incompletely understood. We have previously crossed K-ras^{Val12} transgenic mice with Ah-*Cre* mice to produce K-ras^{Val12}/*Cre* offspring that inducibly express K-ras^{Val12} 4A and 4B in the intestines, but this alone showed no significant effect on intestinal adenoma formation. Here, we crossed these mice with Min mice to evaluate the effect of K-ras^{Val12} and *Apc* mutation on intestinal tumourigenesis *in vivo*. The double mutant K-ras^{Val12}/*Cre*/$Apc^{Min/+}$ mice showed a moderate (1.86-fold) increase in adenomas in the small intestines, but a striking acceleration (6-fold increase) of large intestinal adenoma formation (P<0.01) and significantly reduced survival (by ~5 weeks) compared with control $Apc^{Min/+}$ mice (P<0.01). There was recombination of the mutant K-ras^{Val12} transgene in 80% of large intestinal adenomas with expression of both K-ras^{Val12} 4A and 4B isoform transcripts and expression of K-Ras^{Val12} protein. The large intestinal adenomas showed immunohistochemical evidence of activation of MapK, Akt and Wnt signaling pathways and this was confirmed by quantitative RT-PCR analysis of relative transcript expression levels of target genes using a panel of 23 selected genes evaluated in both adenomas and non-tumour-bearing intestines. Several genes including *Tiam1*, *Gastrin*, *CD44*, *uPA*, *Igfbp4*, *VEGF* and *Cox-2* that are known to be transcriptionally regulated by activation of the Wnt signaling pathway were found to be expressed at higher levels in the large intestinal adenomas from K-ras^{Val12}/*Cre*/$Apc^{Min/+}$ mice compared with those from controls, although other Wnt signaling pathway target genes remained unchanged. These data show that intestinal expression of K-ras^{Val12} accelerates *Apc*-initiated intestinal adenomagenesis *in vivo* with particularly striking tumour promotion in the large intestines and indicate synergistic effects between mutant K-*ras* and mutant *Apc* in this process.

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Introduction

Human colorectal carcinomas accumulate multiple mutations in cancer-related genes, including oncogenes such as *KRAS* and tumour suppressor genes, such as *APC* (*adenomatous polyposis coli*), *SMAD* 2/4, *p53* and others (1,2). *KRAS* mutations can be detected at all stages of colorectal cancer development, from dysplastic aberrant crypt foci (ACF) and hyperplasic polyps to late adenomas and carcinomas (3,4). Mutational activation of the *KRAS* gene reduces or abolishes the protein's intrinsic GTPase activity, locking it in its GTP-bound conformation that results in constitutive activation of MAPK and PI3K/AKT signal transduction pathways (5), modulating cell proliferation, apoptosis, senescence, motility and differentiation (6-10), but its roles in colorectal tumour initiation and progression are still incompletely understood.

The *APC* gene, is known as the 'gatekeeper' of colorectal cancer, and is mutated in >80% of colorectal adenomas and carcinomas. Inherited *APC* defects give rise to the syndrome of familial adenomatous polyposis coli (FAP) manifested by the formation of hundreds of colorectal adenomas (11). The $Apc^{Min/+}$ mouse model harbours a dominant germline mutation of *Apc* at codon 850 of the mouse homologue and displays *Apc*-driven tumourigenesis almost entirely in the small intestines with very few adenomas forming in the large intestines (12). Investigation of adenomas from both FAP patients and $Apc^{Min/+}$ mice (each with one inherited *Apc* gene mutation) has shown that the second allele is almost always mutated or deleted in the adenomas formed. This indicates that mutation or loss of *APC* alleles is the most important alteration for the initiation of intestinal tumourigenesis (13-15).

Although colorectal tumours appear to arise via a multistep process, it is not clear which genetic alterations are required

for the formation of adenomas or whether this process involves the cooperation of specific mutated genes. *KRAS* and *APC* mutations both occur during the early stages of colorectal adenoma formation, including dysplastic aberrant crypt foci, early monocryptal adenomas, oligocryptal adenomas and established adenomas. However, the precise contributions of mutated *KRAS* and *APC* to intestinal tumourigenesis are still elusive. *In vitro* studies have shown that when these genes are both mutated, they promote transformation of colonic epithelial cell lines suggesting synergistic cooperation, however *in vivo* studies are contradictory (16,17).

We have previously generated mice with an inducible K-*ras*^{Val12} transgene and crossed these with Ah-*Cre* mice to produce K-*ras*^{Val12}/*Cre* offspring (18). Following treatment with β-naphthoflavone to induce Cre-mediated transgene recombination, activation of intestinal expression of K-*ras*^{Val12} 4A and 4B had no significant effect on the formation of intestinal adenomas (18). In the current study, we crossed these intestinal inducible K-*ras*^{Val12}/*Cre* mice with *Apc*$^{Min/+}$ mice to investigate whether combined mutation of these two genes can cooperate to modify intestinal tumourigenesis. The double mutant offspring have significantly reduced survival due to increased numbers of intestinal adenomas, particularly in the large intestines, compared with *Apc*$^{Min/+}$ control mice. The data presented here clearly show that mutant K-*ras*^{Val12} accelerates *Apc*-driven large intestinal tumourigenesis *in vivo*.

Materials and methods

Mice. *Apc*$^{Min/+}$ mice have a nonsense mutation at codon 850 in the *Apc* gene (12). K-*ras*^{Val12} mice carry a codon 12 Glycine to Valine mutation and express both K-*ras*^{Val12} 4A and 4B isoform transcripts in the intestines upon β-naphthoflavone-induction of Cre-mediated *LoxP* recombination (18). All animals had free access to standard laboratory diet and water *ad libitum* and were maintained on a 12-h light/12-h dark schedule. The animal experimental protocols were carried out under UK Home Office licence.

Genotyping of mice by polymerase chain reaction (PCR). Tail material was digested overnight at 55˚C in 500 μl DNA lysis buffer. DNA was extracted by standard procedures and amplified in a 30μl PCR reaction. The quality of genomic DNA samples was estimated by amplifying a wild-type *Apc* gene fragment (18). The PCR conditions that were used for genotyping K-*ras*^{Val12} and *Cre* mice were as follows: 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 45 sec for 35 cycles (18,19). The genotyping assays for *Apc*$^{Min/+}$ mice have been described previously (20).

Analysis of intestinal tumours. The total number of intestinal polyps was assessed for each mouse. The whole intestinal tract was removed, rinsed gently in PBS, opened lengthwise, and spread out flat on filter paper. All polyps were counted under a dissecting microscope at x15 magnification and the smallest adenomatous polyps identified were about 0.5 mm in diameter. Tumours were cut in two and half was used for extraction of DNA, RNA and protein and the other half for formalin fixation and histopathological analysis. The tumour samples from the small and large intestines were

processed for paraffin embedding following fixation in 10% neutral-buffered formalin solution and sections were prepared for H&E staining and immunohistochemistry as described previously (21,22).

Analysis of LoxP site recombination by PCR and DNA sequencing. In order to induce *Cre* expression via the *Ah* promoter, the *Ah-Cre* containing mice were injected with 80.0 mg/kg β-naphthoflavone (β-NF) (Sigma, Dorset, UK) dissolved in corn oil for 6 days as described previously (19). Genomic DNA was prepared from intestinal tumours and non-tumour-bearing intestinal tissue samples by overnight proteinase K digestion and purification using a QIAamp Tissue kit (Qiagen, UK). Transgene recombination was detected by PCR using the primers: RE-CMV, 5'-TCAGATCACTAGAAGCTTTATTGCGG-3' (sense primer), which is located in the CMV promoter; and RE-K-*ras*^{Val12}, 5'-TTCTGAATTAGCTGTATCGTCAAGGC-3' (antisense primer), which is located in exon 3 of the K-*ras* transgene to generate a ~500 bp PCR product (18). The PCR assay detected Cre-mediated recombination of the two *LoxP* sites that flank the 'Stop' cassette. Following removal of the 'Stop' cassette by recombination, the CMV promoter lies immediately adjacent to the transgene and drives expression of the K-*ras*^{Val12} 4A and 4B transcripts. In some experiments the amplified PCR product was DNA sequenced by standard methods to demonstrate loss of the 'Stop' cassette and retention of only a single *LoxP* site between the CMV promoter and the human K-*ras*^{Val12} transgene.

Analysis of expression of K-ras^{Val12}. Human K-*ras*^{Val12} 4A expression was determined using the primer pair K-*ras* 4A upstream primer (5'-AGTGCAATGAGGGACCAGTACATGAGG-3'), located in K-*ras* exon 3, and K-*ras* 4A downstream primer (5'-TTTGCTGATGTTTCAATAAAAGGAATT-3') located in K-*ras* exon 4A. Human K-*ras*^{Val12} 4B expression was determined using the K-*ras* 4B upstream primer (5'-GTACCTATGGTCCTAGTAGGAAATAAA-3'), also located in human K-*ras* exon 3; and K-*ras* 4B downstream primer (5'-CTGATGTTTCAATAAAGGAATTCCA-3') located in human K-*ras* exon 4B. The PCR product sizes for K-*ras* 4A and 4B were 248 and 159 bp, respectively. For quantitative RT-PCR, 100 ng of total RNA of different tissue samples was reverse transcribed in 25 ml volume using the iTaq SYBR Green kit RT-PCR kit (Bio-Rad, UK). RT-qPCR was performed using the following PCR cycling conditions: 95˚C for 3 min, followed by 45 cycles of 95˚C for 15 sec and 60˚C for 1 min.

Western blotting and immunohistochemical analysis. For Western blot analysis, fresh tissue samples were lysed in protein lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40 and protease inhibitors. Soluble protein lysates were quantified using the BCA Protein Assay kit (Pierce, UK). Total proteins were separated by 15% SDS-PAGE, transferred to nitrocellulose membranes (Schleicher and Scheull, UK), and incubated with the corresponding antibodies including anti-K-Ras^{Val12} (Oncogene Co., USA), and anti-Cre rabbit polyclonal antibody (Novagen, USA). Blots were hybridized with antibodies in 1:1000 dilutions and visualization was performed with chemiluminescence. The pMAPK Family Rabbit mAb Sampler Kit and the pAKT Pathway Sampler Kit (Cell Signaling, USA) were used for MAPK and AKT pathway

Table I. PCR primer sequences.

Gene	Upstream PCR primer	Downstream PCR primer
Axin	TCTCCGAGACAGAGACAAAATCAC	TCTTGGTTAGCAGCTCCTTGAACT
Apc	TGATACTTCTTCCAAAGCTTTGGCTAT	TCTCGTTCTGAGAAAGACAGAAGCT
β-catenin	CCATTGAAAATATCCAAAGAGTAGC	CTCAGACATTCGGAATAGGACAG
CD44	GCGGTCAATAGTAGGAGAAGGTGT	CTCGTCAGCTGTCATACACTGGT
CMV	TGACGTCAATGGGTGGAGTA	TGCCAAAACAAACTCCCATT
C-myc	AAATCCTGTACCTCGTCCGATTC	ATCAATTTCTTCCTCATCTTCTTGC
Cox-2	ACAGACTGTGCCACATACTCAAGC	GATACTGGAACTGCTGGTTGAAAAG
Cyclin D1	TTTCTTTCCAGAGTCATCAAGTGTG	ACCAGCCTCTTCCTCCACTTC
Cyclin D2	GAACTGGTAGTGTTGGGTAAGCTG	GTACATGGCAAACTTGAAGTCG
E-cadherin	TACCCGGGACAATGTGTATTACTA	GAAGTTTCCAATTTCATCAGGATT
EGFP	GCAAGGGCGAGGAGCTGTTC	CCATGCCGAGAGTGATCCCG
Ephb2	CCATTGAACAGGACTACAGACTACC	CACCGTGTTAAAGCTGGTGTAG
Gastrin	ACCAATGAGGACCTGGAACAG	TGCTAGTCCTACTGGTCTTCCTCA
Igfbp4	CACGAAGACCTCTTCATCATCC	CCTAGTAGGGGGCACTGAGTC
K-ras 4B	TCTTAAGGCATACTAGTACAAGTGGT	TTTGTTTCACACCAACATTCA
MMP7	GAGTACTGGACTGATGGTGAGGAC	CATATAACTTCTGAATGCCT
Neo	TGGAGAGGCTATTCGGCTATGACTGGG	TGGATACTTTCTCGGCAGGAGCAAGGTG
PP1A	CTACTGTGTGATCTCCTGTGGTCT	AGAAGAGTGTCACCAACTGTCTCTT
PP2A	GAATGACTACACTCTTCTGCATCAA	TATCAAGAATGGGTCCTATCTTCTG
P21	CTGTCTTGCACTCTGGTGTCTG	GGCACTTCAGGGTTTTCTCTT
P70S6K	ATCTGAAGAGGATGTGAGTCAGTTT	TGTTCGTGGACTACCAATAAATCTT
Pem	GAGTCAAGGAAGACTCGGAAGA	GGCCTTTTCCTCCATTTAATTC
PPAR δ	CAAGTTCGAGTTTGCTGTCAAGTT	GACCTGCAGATGGAATTCTAGAGC
TCF-4	ACCATGTTGATCACAGACACCAA	GCTGCAGGTGCTGGATGTT
Tcl-1	CAAGAGTAATGAAAAATTCCAGGTG	GATATGGTACAGGATCTGCCAATAC
Tiam1	AATTGTCCACGTGAAATCAGAGT	CTTTAAGCGCACACAATCTCTTG
Trap1a	AAGAATTGGAGAACCTGATGGA	GGGTCGTGGAAGAAATAAATCA
uPA	ATCTTGCACGAATACTACAGGGAAG	CAGTGATCTCACAGTCTGAACCAAA
VEGF	GGAGTACCCCGACGAGATAGAGTA	GAAGCTCATCTCTCCTATGTGCTG

protein analysis by Western blotting. Immunohistochemical analysis was performed with the goat anti-Gastrin polyclonal antibody (Santa Cruz Biotechnology, USA), the rabbit anti-Cox-2 monoclonal antibody and the rabbit anti-vascular endothelial growth factor (VEGF) polyclonal antibody (both from Novus Biologicals, USA) as described previously (21,22).

RT-qPCR analysis. Real-time quantitative reverse transcription PCR (real-time RT-qPCR) was carried out to measure the relative expression levels of selected target genes (Table I shows PCR primer sequences) using the comparative Ct method that has been described previously (18,20-22). Gene expression levels were normalized against β-actin (using the following formulas: $\Delta\Delta Ct = \Delta Ct$ test-ΔCt control, fold change $= 2^{-\Delta\Delta Ct}$).

Statistical analysis. The SPSS statistical package was used for all statistical analyses. Student's t-tests were used to compare adenoma numbers and Kaplan-Meier curves were used to estimate the time-related probabilities of survival among the different mouse cohorts. The log-rank test was used to estimate the significance of differences between survival distributions.

Results

Intestinal tumour formation is increased in K-ras^{Val12}/Cre/Apc$^{Min/+}$ mice. Apc$^{Min/+}$ mice on a Black6 background typically develop 30-50 intestinal adenomas per mouse in the first 6 months of life, with the vast majority in the small intestines and very few in the large intestines. To assess the effects of expression of K-ras^{Val12} on intestinal tumourigenesis initiated by mutant *Apc*, K-ras^{Val12}/Cre mice were crossed with Apc$^{Min/+}$ mice to generate a cohort of K-ras^{Val12}/Apc$^{Min/+}$/Cre mice (n=26). Four weeks after birth, β-NF was injected intraperitoneally for 6 days to induce transient intestinal Cre expression to activate the K-ras^{Val12} transgene in the small and large intestines. Two control cohorts of *Cre* mice (n=29) and Apc$^{Min/+}$ or Apc$^{Min/+}$/Cre mice (n=25) were also treated in the same way. Some
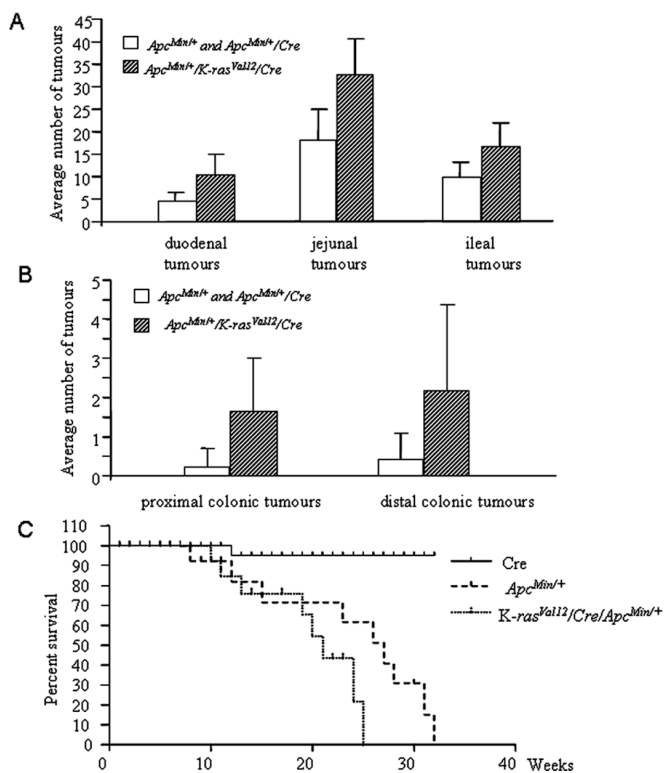
Figure 1. Intestinal tumour frequency and lifespan of $Apc^{Min/+}$ and K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice. (A, B) Average number of small intestinal tumours (A) and large intestinal tumours (B) by location, in K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice (cross-hatched bars), compared with control $Apc^{Min/+}$ and $Apc^{Min/+}$/Cre mice (open bars) (error bars, standard deviation). (C) Kaplan-Meier survival curves of Cre mice (n=29), $Apc^{Min/+}$ mice (n=25 in total, including 16 $Apc^{Min/+}$ and 9 $Apc^{Min/+}$/Cre mice) and K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice (n=26). Ages of the animals at death (or when sacrificed if moribund) are given in weeks (x-axis).

of the $Apc^{Min/+}$ or $Apc^{Min/+}$/Cre mice developed a few colonic adenomatous polyps, but most of the adenomas were found in the small intestines as expected. The double mutant K-ras^{Val12}/$Apc^{Min/+}$/Cre mice showed reduced survival due to increased formation of multiple intestinal adenomas (Figs. 1 and 2), with bowel obstruction and/or intestinal haemorrhage, and their average lifespan decreased from 20.9±4.7 weeks (mean ± SD) for $Apc^{Min/+}$ mice to 15.2±3.8 weeks for K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice (P<0.01), with no differences between males and females (Fig. 1). The number of duodenal adenomatous polyps increased from 4.56±1.99 (mean ± SD of adenomas per mouse in $Apc^{Min/+}$ mice) to 10.54±4.50 (in K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice, P<0.01); the number of jejunal tumours increased from 17.94±6.96 (in $Apc^{Min/+}$ mice) to 32.54±8.07 (in K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice, P<0.01); and the number of ileal adenomas increased from 9.75±3.51 (in $Apc^{Min/+}$ mice) to 16.82±5.04 (in K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice, P<0.01). Overall, there was a 1.86-fold increase in the total number of small intestinal adenomas in the K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice compared with $Apc^{Min/+}$ mice. In the large intestines, the number of proximal colonic adenomas increased from 0.22±0.48 (in $Apc^{Min/+}$ mice) to 1.64±1.36 (in K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice, P<0.01); and the number of distal colonic adenomas increased from 0.41±0.68 (in $Apc^{Min/+}$ mice) to 2.18±1.77 (in K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice, P<0.01) (Fig. 1), showing an overall 6-fold increase in the total

number of large intestinal adenomas in the K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice compared with $Apc^{Min/+}$ mice. All small and large intestinal tumours were shown to be moderately dysplastic adenomas histopathologically with no evidence of invasive malignancy (Fig. 2).

The K-ras^{Val12} transgene is recombined and expressed in the intestinal adenomas of K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice. A PCR assay was used to detect recombination occurring at the two *LoxP* sites (flanking the 'Stop' cassette) in the K-ras^{Val12} transgene, in 8 out of 10 large intestinal tumours (Fig. 3A). This was confirmed by DNA sequencing of the PCR products (data not shown) which showed that the 'Stop' cassette had been deleted between the two *LoxP* sites, leaving only one *LoxP* site between the CMV promoter and the adjacent K-*ras* sequence and confirmed the presence of the G12V mutation in the transgene. Recombination-positive large intestinal tumours from K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice were further tested to demonstrate the expression of K-ras^{Val12} 4A and 4B isoform transcripts by RT-PCR analysis, and K-Ras^{Val12} protein expression by Western blot analysis using specific anti-K-Ras^{Val12} antibody (Fig. 3B and C).

Evidence of activation of the MapKinase, Akt and Wnt signaling pathways in adenomas from K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice. Both immunohistochemistry (Fig. 4) and real-time quantitative reverse-transcription PCR (Table II and Fig. 5) were used to detect changes in protein and RNA expression in intestinal adenomas and compare the relative mRNA expression levels of a selected panel of 23 genes between non-tumour-bearing morphologically normal large intestine and large intestinal tumours from K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice and $Apc^{Min/+}$ or Cre/$Apc^{Min/+}$ or K-ras^{Val12}/Cre control mice. Activation of mutant K-*ras* in the adenomas from K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice was associated with immunohistochemical evidence of increased pERK1/2 and pAkt, indicating activation of the MapK and Akt pathways, respectively. Immunohistochemical assessment also showed increased nuclear localisation of β-catenin, consistent with enhanced signaling of the Wnt pathway in these adenomas and this is in keeping with increased immunohistochemical expression of the Wnt-target gene *VEGF* (Fig. 4). Quantitative reverse-transcription PCR showed increased relative transcript levels of the stem cell associated genes *Trap1a* and *Tcl-1* (10) in large intestinal tumours from K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice compared with tumours or normal colonic tissues from control mice. These qRT-PCR data also showed significant increases in Wnt or MapK pathway target genes such as *Tiam1*, *Gastrin*, *CD44*, *uPA*, *Igfbp4*, *VEGF* and *Cox-2* in large intestinal adenomas from K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice compared with adenomas or normal colonic tissues from control mice. Some *Wnt* signaling pathway target genes such as *p70S6K* and *cyclin D1* remained relatively unchanged in K-ras^{Val12}/Cre/$Apc^{Min/+}$ murine large intestinal tumours (Table II and Fig. 5).

Unsupervised hierarchical clustering analysis of the relative transcript levels of the 23 genes that were differentially expressed between the colorectal tumours from K-ras^{Val12}/Cre/$Apc^{Min/+}$, K-ras^{Val12}/Cre, Cre/$Apc^{Min/+}$, Cre and $Apc^{Min/+}$ mice showed grouping together of the large intestinal adenomas with those from K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice clustered on the right of the heatmap and those from $Apc^{Min/+}$ or Cre/$Apc^{Min/+}$ mice mostly on the left of the heatmap, with grouping of normal
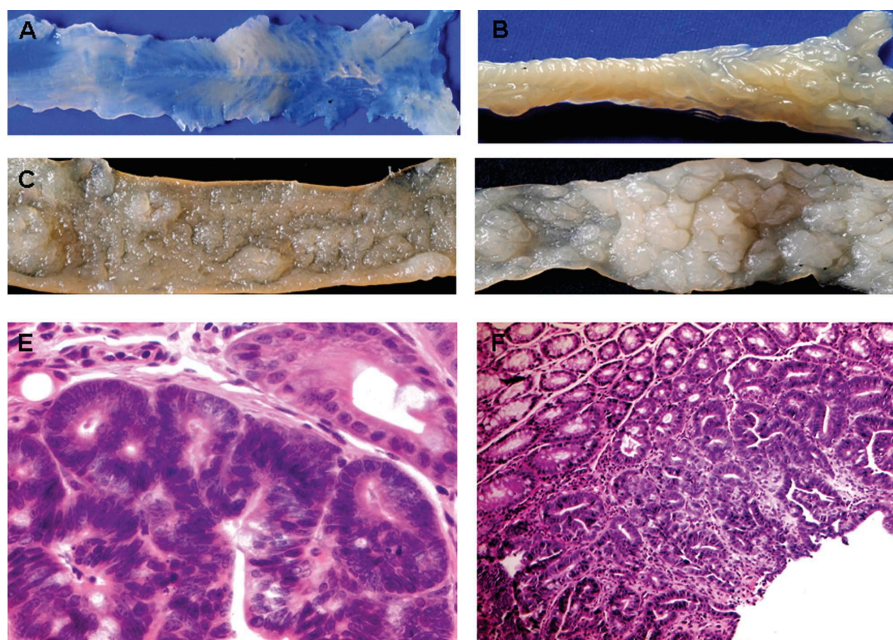
Figure 2. Tumour development in $Apc^{Min/+}$ and K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice. Macroscopic photographs of $Apc^{Min/+}$ (A, C) and K-ras^{Val12}/Cre/$Apc^{Min/+}$ (B, D) murine large intestines (A, B) and small intestines (C, D), showing many more adenomatous polyps in the latter cohort. Photomicrographs of H&E stained large intestinal adenomas from $Apc^{Min/+}$ mice (E) (moderately dysplastic, showing no invasion, x200) and K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice (F) (similar moderately dysplastic, showing no invasion, x50).

Figure 3. Transgene recombination and expression of K-ras^{Val12} in large intestinal adenomas. (A) Transgene recombination assay was performed by PCR amplification of genomic DNA extracted from adenomas, using primers located in the *CMV* promoter and K-ras^{Val12} exon 3, resulting in a ~500 bp fragment, only if Cre-mediated recombination at the *LoxP* sites had occurred in the K-ras^{Val12} transgene, as seen in the large intestinal tumours LIT1 to LIT4 and LIT7 to LIT10. Amplification of a DNA fragment of the *Apc* gene was used as a control for adequate quality of genomic DNA. (B) RT-PCR analysis of the expression of K-ras^{Val12} 4A and 4B transcript isoforms in selected large intestinal tumours (LIT1-4) from K-ras^{Val12}/Cre/$Apc^{Min/+}$ transgenic mice. RT-PCR amplification of β-actin transcript was used to control the quality of RNA samples. (C) Western blot analysis demonstrating the expression of mutant K-Ras protein using anti-K-Ras^{Val12} protein antibody in selected large intestinal tumours (LIT1-4) from K-ras^{Val12}/Cre/$Apc^{Min/+}$ mice, whereas LIT 5 and 6 showed no transgene recombination or expression.

large intestine tissues from the cohorts in the middle of the heatmap (Fig. 5). This pattern indicates that the combination of mutant K-*ras* and mutant *Apc* modulates gene expression in characteristic ways in these intestinal tumours and suggests that expression of mutated K-*ras* activates the MapK, Akt and Wnt downstream signaling pathways consistent with synergistic effects of mutant K-*ras* and mutant *Apc* on a subset of *Wnt* pathway target genes in adenomas *in vivo*.
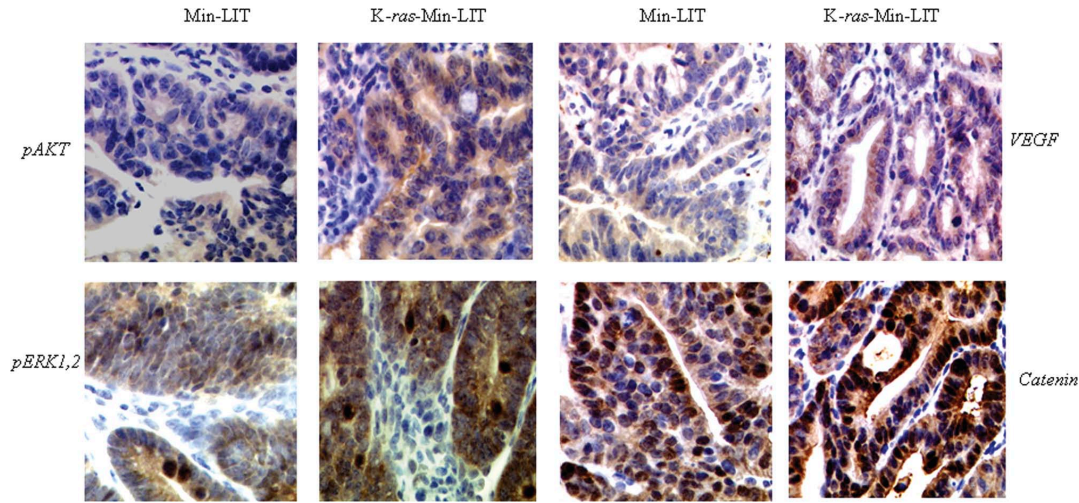
Figure 4. Immunohistochemical analysis of intestinal adenomas from *Apc^Min/+* and K-*ras^Val12*/*Cre*/*Apc^Min/+* mice. Large intestinal tumours from *Apc^Min/+* mice (Min-LIT) and K-*ras^Val12*/*Cre*/*Apc^Min/+* transgenic mice (K-*ras*-Min-LIT) were analysed immunohistochemically for expression of pAKT, pERK1/2, VEGF and β-catenin proteins.
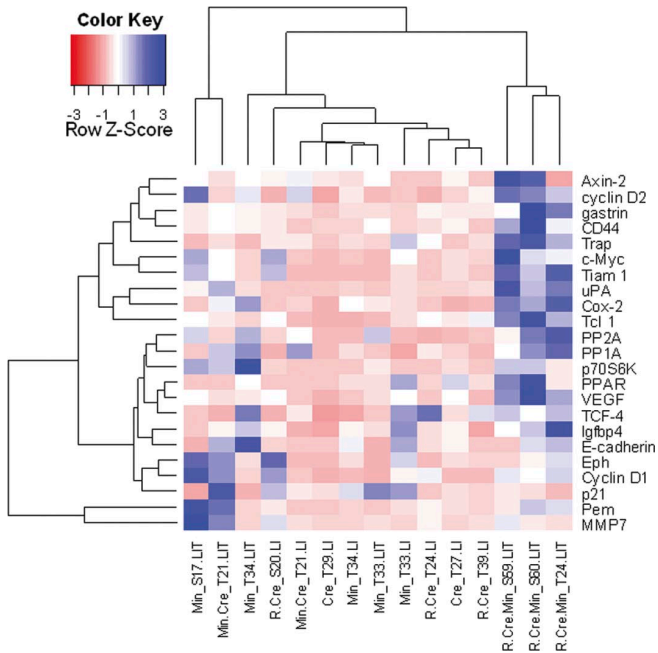

Figure 5. Unsupervised hierarchical clustering analysis of differentially expressed genes in non-neoplastic colonic tissues and large intestinal tumours. Relative RNA expression levels determined by quantitative RT-PCR amplification of 23 selected genes (right) from either morphologically normal large intestinal tissues (LI) or large intestinal tumours (LIT) (bottom), were analysed by unsupervised hierarchical clustering to show grouping of LIT from K-*ras^Val12*/*Cre*/*Apc^Min/+* mice (R.Cre.Min) on the right of the heatmap and LIT from *Apc^Min/+* mice (Min) or *Cre*/*Apc^Min/+* mice (Min.Cre) mostly on the left of the heatmap, with grouping of normal large intestine (LI) tissues from all cohorts in the middle of the heatmap. Red and blue cytobands indicate underexpressed and overexpressed genes respectively (key provided top left).

Discussion

The progression of human colorectal cancer from normal to adenoma to carcinoma involves many genetic and epigenetic

alterations including *APC*, *KRAS*, *BRAF*, *PIK3CA*, *β-catenin*, *SMAD2* and *4* and *TP53* genes amongst others (23,24). *KRAS* mutations have been detected in clusters of abnormal crypts in the colonic mucosa known as aberrant crypt foci (ACF), as well as in early and established adenomas and in cancer-adjacent morphologically normal colon (5,25). One of the commonest mutations that activate the *KRAS* oncogene is the codon 12 glycine to valine mutation and this has been associated with colorectal cancers with a poor prognosis (26). Although activating mutations of *KRAS* are associated with early to intermediate stages of adenoma formation, it is still unclear whether their tumourigenic effects occur only in the context of pre-existing *APC* or other gene mutations (27). The view that the oncogenic effects of mutated *KRAS* depend upon previous *APC* mutations in intestinal cancer has recently been challenged. A study on the mutational status of ACF, which are thought to be putative precursor lesions of colorectal adenomas and cancers, has demonstrated the presence of frequent *KRAS* mutations, in the absence of mutations of *APC* or its downstream target *β-catenin* (28). To address the question whether activated *KRAS* is able to initiate intestinal tumour formation or rather depends on previous *APC* mutations, we developed a *Cre*/*LoxP*-based transgenic system that expresses the Cre recombinase under induction of the *Ah* promoter by β-NF, which drives recombination and expression of the mutant K-*ras* transgene in the murine adult intestinal crypt stem cells with a high degree of spatial-temporal control.

This transgenic line of mice has a single integrated copy of the K-*ras^Val12* transgene and following β-NF-mediated induction we detected only minimal phenotypic changes in the intestines of these mice with just two intestinal tumours in 2/20 mice over a period of two years (one adenoma in the large intestines and one adenoma in the small intestines), while another 8 mice were killed after one year and showed no intestinal tumours (18). This is consistent with the appearance of only a few intestinal tumours in other previous studies (29), and indicates that expression of mutated K-*ras* alone in intestinal epithelium is unable to drive the initiation of intestinal tumour formation. Johnson and

Table II. Relative expression levels for selected genes by real-time quantitative RT-PCR.

Gene	C LI Ave (3)	C LI SD	K/C LI Ave (3)	K/C LI SD	K/C vs. C LI t-test	M LI Ave (3)	M LI SD	M LIT Ave (4)	M LIT SD	K/C/M LIT Ave (3)	K/C/M LIT SD	K/C/M vs. M LIT t-test
CD44	1.52	0.66	1.49	0.64	0.48	1.21	0.22	**2.54**	0.52	**7.02**	4.17	**0.04**
Cyclin D2	1.06	0.59	1.38	0.98	0.37	2.75	1.50	3.39	2.65	6.09	1.26	0.08
PP1A	1.12	0.27	1.26	0.53	0.25	2.34	2.14	2.90	1.91	4.53	1.92	0.16
PP2A	0.88	0.10	0.98	0.45	0.36	1.38	0.94	3.21	1.38	4.82	2.44	0.16
Axin-2	1.80	0.71	1.58	0.58	0.40	2.16	0.99	2.52	0.46	5.50	4.39	0.11
P70S6K	0.95	0.34	0.85	0.19	0.32	0.69	0.13	4.34	2.69	2.99	1.25	0.23
E-cadherin	1.56	0.66	1.56	0.16	0.50	2.78	1.45	3.16	2.68	2.80	1.29	0.42
p21	1.44	0.38	2.08	1.39	0.30	3.07	1.25	3.04	2.84	1.54	0.42	0.21
Igfbp4	1.39	0.97	1.80	0.94	0.29	3.08	2.49	**2.56**	1.27	**5.35**	2.78	**0.06**
Cox-2	0.89	0.11	1.96	0.26	0.01	3.73	1.23	**5.65**	3.83	**12.62**	2.27	**0.02**
TCF-4	1.03	0.31	2.14	1.16	0.10	1.74	1.03	1.64	1.03	2.23	0.40	0.20
uPA	1.42	0.51	1.84	0.88	0.22	1.90	0.77	**4.47**	4.12	**14.28**	4.49	**0.01**
Gastrin	0.93	0.45	1.50	0.19	0.06	0.96	0.19	**1.58**	0.40	**5.99**	3.84	**0.03**
VEGF	1.31	0.60	1.44	0.55	0.41	1.50	0.47	**1.68**	0.26	**4.09**	1.09	**0.01**
Tiam1	1.18	0.92	3.55	3.90	0.21	1.34	1.12	**3.74**	3.40	**11.48**	3.33	**0.02**
Cyclin D1	0.85	0.17	2.43	1.56	0.09	1.31	0.53	3.01	2.55	2.63	0.50	0.41
MMP7	0.60	0.41	3.48	2.65	0.09	0.67	0.46	9.31	10.61	3.15	2.52	0.19
C-myc	1.10	0.70	3.39	3.51	0.20	1.76	1.27	3.35	2.97	7.68	5.00	0.10
PPAR	1.46	0.97	0.97	0.24	0.24	1.72	1.36	**1.13**	0.56	**3.53**	2.09	**0.04**
Eph	1.12	0.57	3.00	3.51	0.24	2.06	1.63	3.79	3.16	2.41	1.13	0.26
Pem	1.31	1.08	4.11	2.34	0.04	2.12	1.51	15.30	17.21	10.60	5.33	0.34
Tcl-1	1.77	1.81	5.91	0.43	0.04	1.28	1.26	**3.08**	2.13	**14.77**	4.52	**0.01**
Trap1a	0.69	0.27	2.38	1.00	0.06	3.23	2.43	**0.93**	0.84	**9.75**	2.68	**0.01**

C, *Cre* genotype; K/C, K-*ras*^{Val12}/*Cre* genotype; M, *Apc*$^{Min/+}$ genotype; K/C/M, K-*ras*^{Val12}/*Cre*/*Apc*$^{Min/+}$ genotype; LI, large intestine; LIT, large intestinal tumour; Ave, average value; (n), number of samples analysed given in brackets; t-test, Student's t-test P-value; statistically significant Student's t-test P-values highlighted in bold.

colleagues used a transgenic model, in which the activation of mutant K-*ras* was generated at random by spontaneous recombination occurring within the allele and showed that these mice were predisposed to a range of tumour types, predominantly early onset lung adenocarcinomas, but no intestinal tumours (16). Janssen and colleagues have also generated mice with a mutated K-*ras* gene driven by the regulatory region of the murine *villin* promoter, however these *villin*-K-*ras*^{Val12} mice developed just two tumours over a 6 month period (29). The *in vivo* oncogenic activity of K-*ras* appears to be dependent on cellular context (30) and requires other additional mutations in order to contribute to intestinal tumourigenesis, such as mutant *Apc* or defective mismatch repair gene *Msh2* (18). Johnson and colleagues produced a double mutant mouse model that carried both a mutated K-*ras* allele and the *Apc*$^{Min/+}$ allele, but the tumour type and frequency found in these mice did not change. However, it is not clear whether K-*ras* was activated in the intestinal tumours that had formed in these mice. Sansom and colleagues did show synergistic co-operation between mutant

K-*ras* and mutant *Apc*, mostly in kidney tumour formation but also in intestinal tumourigenesis, although this study did not detect any differences in the activation of the Raf/MEK/ERK pathway in the intestinal tumours (17). Here, we have shown that mutated K-*ras* significantly accelerates intestinal adenomagenesis on a background of inherited *Apc* mutation, with a 6-fold increase in adenomas in the large intestines, with significantly reduced survival. We have confirmed this result for mice bearing a K-*ras*^{Val12} transgene in the current study as well as mice with a K-*ras*^{Asp12} transgene in our previous study (20), and there was no significant difference in the intestinal oncogenic effect between these two types of K-*ras* mutation.

Previous studies have shown that expression of mutant K-*ras*^{Val12} in either mouse embryonic stem (ES) cells or mutant K-*ras* transgenic mice was associated with changes in expression of a range of gene transcripts and proteins, including stem cell associated genes and components of the Mapk and Akt pathway genes (10,18,20-22). Quantitative RT-PCR and immunohistochemical expression analysis of the intestinal tumours

that developed in the K-*ras*^{Val12}/*Cre*/*Apc*$^{Min/+}$ mice showed that mutant K-*ras* modulates the expression of many downstream effectors of the MapK, Akt and Wnt signaling pathways. One characteristic example is that of the *VEGF* gene, a known target gene of Wnt pathway activation (31), which shows further enhanced expression in adenomas from K-*ras*^{Val12}/*Cre*/*Apc*$^{Min/+}$ mice. *VEGF* is a key regulator of tumour angiogenesis, a process which is not exclusive to advanced stages of tumour development, but is also observed in benign intestinal adenomas. Similarly, K-*ras*^{Val12} has been shown to induce the activity of *TCF-4* promoter in Caco-2 and HeLa cells, leading to an increase in the stability of *β-catenin*, with nuclear translocation and *β-catenin*/*TCF-4* complex formation.

There are multiple lines of evidence that demonstrate synergism between the MapK/Erk and Wnt signaling pathways. Induction of K-*ras*^{Val12} has been shown to stimulate Wnt signaling in colonic cancer through inhibition of *GSK-3β* (32), which is regulated by the activation of the PI3K/Akt pathway. Wnt3a stimulation of G1/S phase cell cycle progression was reduced after treatment with an Erk inhibitor, suggesting that Wnt3a could promote proliferation via an Erk-dependent pathway (33). The expression of *Gastrin*, as a tumour growth factor, is significantly increased in some colonic cancers compared with their corresponding normal colonic mucosa and is known to be induced upon activation of the Raf-MEK-ERK signal transduction pathway. Interestingly, the Wnt signaling pathway moderately stimulates the *Gastrin* gene promoter, and Chakladar and colleagues (34) found a strong (25- to 40-fold) synergistic stimulation of the *Gastrin* promoter by the combination of oncogenic *β-catenin* and K-*ras* overexpression. *Gastrin* promoter activation could be further enhanced or suppressed by the co-expression of wild-type *SMAD4* or a dominant negative mutant of *SMAD4*, supporting co-operation between oncogenic Wnt and Ras signaling pathways inducing enhanced *Gastrin* expression (34). *In vivo*, *Apc*$^{Min/+}$ mice that overexpress one of the alternatively processed forms of Gastrin, known as glycine-extended Gastrin, show a significant increase in intestinal adenomatous polyp number. On the contrary, *Gastrin*-deficient *Apc*$^{Min/+}$ mice showed a marked decrease in intestinal polyp number and a significantly decreased adenoma proliferation rate (33). The immunohistochemical and RT-PCR expression data also demonstrated that adenomas from K-*ras*^{Val12}/*Apc*$^{Min/+}$ mice showed increased expression of *Gastrin*.

The T lymphoma invasion and metastasis 1 gene (*Tiam1*) product has been suggested to be an important effector pathway for some of the effects of mutated K-*ras*. *Tiam1*$^{-/-}$ mice were shown to be resistant to the development of mutant *ras*-induced skin tumours following treatment with the carcinogen 7, 12-dimethylbenzanthracene and promoter 12-O-tetra-decanoylphorbol-13-acetate. Moreover, the few tumours that were detected in *Tiam1*$^{-/-}$ mice grew more slowly than the tumours that were found in wild-type mice. *Tiam1*-deficient mouse embryonic fibroblasts were also resistant to *ras*^{Val12}-induced foci formation. Hence, *Tiam1* was shown to be a critical regulator of *ras*-induced tumour formation (35). Interestingly, *Tiam1* is also a Wnt-responsive gene that is expressed in the base of intestinal crypts and is found to be up-regulated in the intestinal adenomas of *Apc*$^{Min/+}$ mice and human colonic adenomas. Comparison of polyp formation in *Apc*$^{Min/+}$ mice expressing or lacking *Tiam1*, showed that *Tiam1* deficiency significantly reduced the formation of intestinal polyps *in vivo* (35). Here, we showed that adenomas from mutated K-*ras* and *Apc*$^{Min/+}$ mice showed increased *Tiam1* expression, in support of interaction between the K-ras-Tiam1-Rac and canonical Wnt-signaling pathways. The RT-PCR expression data also demonstrated that mutated K-*ras* co-operates with *Apc*$^{Min/+}$ to induce increased expression of *Cox-2* in adenomas *in vivo*, which is consistent with previous findings that co-expression of both mutant *β-catenin* and K-*ras* increased Cox-2 protein expression levels *in vitro* (36).

Overall, this study shows that whereas intestinal expression of K-*ras*^{Val12} alone does not significantly initiate adenoma formation, when combined with mutant *Apc*, the double mutant K-*ras*^{Val12}/*Cre*/*Apc*$^{Min/+}$ mice show a dramatic acceleration of intestinal adenomagenesis, which is associated with significantly reduced survival, due to the 6-fold significant increase of adenomas in the large intestines and the 1.86-fold increase in the small intestines. Tumour expression analysis showed that expression of mutant K-*ras* in adenomas from K-*ras*^{Val12}/*Cre*/*Apc*$^{Min/+}$ mice was associated with increased expression of many genes that are known to be regulated by the MapK, Akt and Wnt signaling pathways, including *Tiam1*, *Gastrin*, *CD44*, *uPA*, *Igfbp4*, *VEGF* and *Cox-2* as well as increased nuclear accumulation of *β-catenin*, consistent with synergism between Wnt and K-Ras signaling pathways.

Acknowledgements

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