

# p53 expression and K-ras mutation in colorectal adenomas

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## Abstract

The frequency of p53 overexpression and K-ras codon 12 mutation was investigated in a series of colorectal adenomas. p53 was detected by immunohistochemistry in only 5% of tumours, whereas K-ras mutation was found in eight of 30 adenomas examined. In vitro, mutant p53 and ras genes cooperate to transform primary rat cells into a tumorigenic cell line. The presence of both p53 overexpression and K-ras mutation in a benign tubulovillous polyp in the present series suggests that in vivo this combination of events is insufficient to cause malignant transformation of a large bowel adenoma.

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A range of genetic alterations have recently been described in colorectal cancer and its benign precursor, the large bowel adenoma. Some of these changes seem to occur at an early stage of the neoplastic process. Hypomethylation of DNA, chromosome 5q deletion, and mutations in the newly identified MCC and APC genes have been identified in both adenomas and carcinomas suggesting that these are early events in the adenoma-carcinoma sequence.<sup>1-4</sup> Conversely, deletions affecting the DCC locus on chromosome 18q and deletions in the region of the p53 gene on 17p are found preferentially in carcinomas and therefore seem to be involved in the later stages of large bowel tumourigenesis.<sup>2</sup> p53 gene mutation or deletion seems to be an important and common event not only in colorectal carcinogenesis but also in many other human tumours. Raised levels of p53 have been described in 44% of 67% of large bowel cancers,<sup>5-9</sup> 36% of breast carcinomas,<sup>10</sup> and 70% of lung tumours,<sup>11</sup> as well as in malignant gliomas, bladder carcinoma, oesophageal carcinoma, ovarian carcinoma, and mesothelioma. In a previous study we described overexpression of the p53 protein in 42% of 52 colorectal carcinomas, using the immunohistochemical detection of p53 as a marker of gene mutation.<sup>12</sup> The validity of this approach has been borne out by other studies combining immunocytochemistry with DNA sequencing that have found accumulation of protein to be invariably associated with the presence of a mutant p53 allele.<sup>7 11 13</sup>

p53 mutants are well known to be capable of cellular immortalisation, and act as nuclear oncogenes in a manner similar to c-myc.<sup>14 15</sup> This action of the mutant protein is in contrast with the recently established tumour suppressor activity of the wild type protein.<sup>16</sup> As well as transformation of established 3T3 cells, p53 will also cooperate with mutant ras genes to transform primary rat cells.<sup>17 18</sup> In at least one study

transformed cells harbouring mutant p53 and ras genes were capable of growing in syngeneic laboratory animals, eventually leading to their death.<sup>17</sup> Because point mutations in K-ras and p53 are common events in colorectal carcinoma, occurring in up to 40% and 67% of tumours respectively, it would be of interest to determine whether p53 and K-ras also cooperate in vivo to produce invasive carcinoma.

The aim of this study therefore was to investigate the frequency with which p53 is overexpressed in large bowel adenomas; to define which, if any, of these adenomas also contain a mutant K-ras gene; and to relate these findings to the presence of malignant change.

## Materials and methods

### SAMPLES

Fresh tissue was available from 38 sporadic adenomas (33 patients), together with two small tubular adenomas from a patient with familial adenomatous polyposis (FAP). Fifteen of the sporadic adenomas were less than 2 cm in size, 16 were greater than or equal to 2 cm in size, and seven had undergone focal malignant transformation, characterised by invasion of neoplastic epithelium through the muscularis mucosae. In the case of malignant polyps only adenomatous mucosa distant from the invasive tumour was selected for analysis. One of us (NS) classified and graded the adenomas according to criteria described by Konishi and Morson.<sup>19</sup>

### p53 IMMUNOHISTOCHEMISTRY

Immunohistochemistry for p53 was performed with a standard technique previously described.<sup>12</sup> Briefly, 4 µm thick frozen sections were fixed in acetone at 4°C for 15 minutes, washed in TRIS buffered saline and incubated successively with primary anti-p53 antibody, biotinylated rabbit anti-mouse immunoglobulin, and streptavidin peroxidase. Antibody binding was visualised with amino-ethylcarbazole. All 40 adenomas were incubated with Pab 421 and 1801, monoclonal antibodies raised against different epitopes found on both wild type and mutant p53 protein. Sixteen specimens were also stained with Pab 240, a novel antibody that binds specifically to the mutant conformation.<sup>20</sup> Each set of sections included a positive colorectal carcinoma control, and a negative control in which the primary antibody was omitted.

### K-RAS MUTATION ANALYSIS

Point mutations in the first two base pairs of K-ras codon 12 were sought by a method pre-

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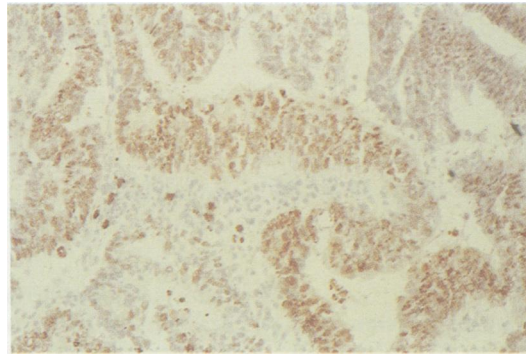


Figure 1: Adenoma showing heterogenous immunohistochemical staining for p53 (original magnification  $\times 40$ ).

viously described,<sup>21</sup> and modified from the non-radioactive restriction enzyme technique of Jiang *et al.*<sup>22</sup> Mutations in codons 13 and 61, which occur much less often in large bowel tumours, were not analysed in this preliminary study. Thirty adenomas were examined. DNA for polymerase chain reaction (PCR) was extracted from sections of fresh tissue immediately adjacent to those used for immunohistochemistry. Three to five 10  $\mu\text{m}$  sections were incubated with 2 mg/ml proteinase K and 1% sodium dodecyl sulphate for 3 days at 37°C. The sample was then extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (24:1). After ethanol precipitation of the aqueous phase at  $-20^\circ\text{C}$  DNA was recovered by centrifugation, dried, and resuspended in distilled water.

With primers described by Jiang *et al* and synthesised on an Applied Biosystems DNA synthesiser a 157 bp fragment of the K-ras gene was amplified containing codon 12. Details of the

#### K-ras codon 12 mutations in 30 large bowel adenomas

Adenoma	No	K-ras mutation (%)
Size:		
<2 cm	14	2 (14)
$\geq 2$ cm	12	4 (33)
Malignant*	4	2 (50)
Mild dysplasia	10	0 (0)
Moderate dysplasia	12	4 (33)
Severe dysplasia	8	4 (50)
Total	30	8 (27)

\*Adenomas showing focal malignant change.

polymerase chain reaction are given in a previous publication.<sup>21</sup> A negative control containing water instead of DNA was included in each reaction. The amplified sequence was visualised on a 2% ethidium bromide stained GTG agarose gel (FMC Bioproducts, Rockland ME, USA) before proceeding to restriction enzyme digestion.

Sixteen microlitres of the amplified PCR product was digested with BstN1 in a total volume of 25  $\mu\text{l}$  under conditions recommended by the suppliers (Boehringer, Mannheim UK, Lewes, Sussex). The digestions were incubated at 60°C overnight and the products analysed on a 3% ethidium bromide stained Nu sieve agarose gel (FMC Bioproducts). Each digestion included a positive colorectal carcinoma or adenoma control known to contain a mutation at codon 12.

#### Results

Only two out of 38 sporadic adenomas were found to overexpress p53 protein. One of these was a 2 cm diameter tubulovillous adenoma exhibiting severe dysplasia, and the other was an 8 cm diameter villous adenoma that had undergone focal malignant change. Unfortunately fresh tissue was not available from the carcinoma, and therefore it was impossible to say whether it also contained a p53 gene mutation. Staining in the two polyps was nuclear and positive with all three p53 antibodies, confirming the presence of a mutant protein phenotype. The distribution of staining was more heterogenous than that previously described in carcinomas with several areas of the polyp failing to express detectable levels of p53 protein. Figure 1 shows the strong nuclear staining seen in one of these adenomas. Neither of the two 0.5 cm diameter FAP adenomas overexpressed p53.

K-ras codon 12 mutations were found in a total of eight out of 30 sporadic adenomas examined. Figure 2 shows a typical BstN1 digest containing examples of both mutant and wild type K-ras alleles. Mutations were notably more frequent in larger adenomas, in adenomas that had undergone focal malignant transformation, and in adenomas exhibiting severe dysplasia (Table). Of particular interest was the coexistence of a mutant K-ras allele and p53 overexpression in the 2 cm tubulovillous polyp described earlier. By contrast the single malignant polyp identified as expressing a mutant type p53 protein did not seem to contain a mutation at codon 12 of the K-ras gene, although obviously we cannot currently exclude the presence of codon 13 or 61 mutations.

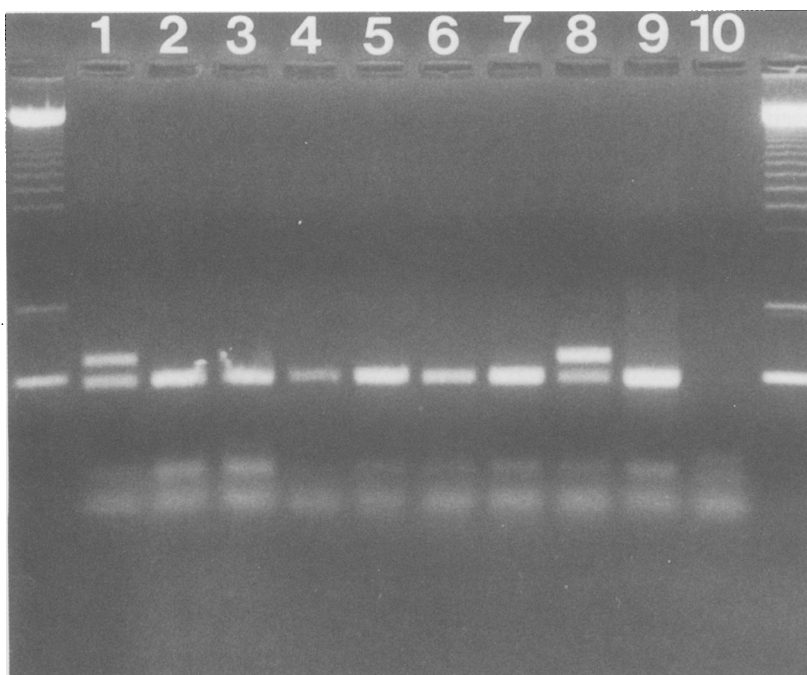


Figure 2: Agarose gel stained with ethidium bromide to show band pattern of mutant and wild type K-ras alleles after Bst N1 digestion. Lanes 1 and 8 contain codon 12 mutants in which a potential restriction site has been lost. Lanes 2-7 and 9 contain wild type K-ras only. Lane 10 is a negative control containing water instead of DNA.

## Discussion

Overexpression and mutation of the p53 gene is now well described in large bowel cancer. There are few reports, however, of p53 gene alterations in the precursor adenoma. Van den Berg *et al*<sup>6</sup> studied 74 adenomas, including 12 FAP polyps, and found that 10% of the sporadic and 17% of the FAP adenomas overexpressed p53. No clear relation was found with degree of dysplasia, and no information was given pertaining to size or presence of malignant change. Napier *et al*<sup>23</sup> described p53 staining in one of 11 (9%) adenomas, Rodrigues *et al*<sup>7</sup> in none out of 10, and Campo *et al*<sup>8</sup> found increased expression in two out of 18 adenomas (11%). More recently Shaw *et al*<sup>9</sup> have sequenced exons 5, 6, and 7 of the p53 gene in 19 polyps without finding any mutations whereas Baker *et al* reported point mutations in 10% of 19 adenomas retaining both p53 alleles.<sup>24</sup> Our finding in this study that only 5% of sporadic adenomas express detectable levels of p53 protein is clearly in line with the results of these other workers, and the use of three different anti-p53 antibodies including 'mutant specific' Pab 240, provides further evidence that the protein detected is a mutant form.

The use of immunohistochemical staining as a marker of point mutation has been critically reviewed by Wynford-Thomas.<sup>25</sup> Although inconsistencies clearly exist, most studies of large bowel tumours show a very strong correlation between immunocytochemical positivity and p53 mutation. Rodrigues *et al* found six of six colorectal cancer cell lines overexpressing p53 to contain a point mutation, and Purdie *et al* described complete agreement between staining with Pab 240 and presence of a mutation in 12 primary large bowel cancers.<sup>7, 26</sup>

It is of interest that the positive tumours in the present series were both 'large' polyps  $\geq 2$  cm in size, and that one had undergone focal malignant transformation. The rarity of p53 gene mutation in adenomas and its prevalence in carcinomas, suggests that p53 mutation is one of the late events in the adenoma-carcinoma sequence, occurring either at the adenoma carcinoma interface or during progression of established cancers. This distribution of p53 gene mutation between adenomas and carcinomas mirrors that of chromosome 17p deletion, believed to signal loss of a wild type p53 allele.<sup>2</sup> Inactivation of one p53 allele by mutation, and the other by deletion, may be important for colorectal carcinogenesis in the same way that deletion of the Rb gene is necessary for the development of retinoblastoma. This interpretation is supported by the demonstration of tumour suppressor activity of wild type p53 in at least two *in vitro* systems,<sup>16, 27</sup> and the finding that reintroduction of the wild type p53 gene into a colorectal carcinoma cell line with one p53 gene mutated and the other deleted, results in suppression of growth.<sup>28</sup>

K-ras gene mutations, by contrast with p53, clearly occur at an earlier stage in the neoplastic sequence. Vogelstein *et al*<sup>2</sup> have described ras mutations in 12% of sporadic adenomas  $< 1$  cm in size, 20% of tumours 1 to 2 cm in size, 100% of adenomas  $> 2$  cm, and in 60% of adenomas that contain a carcinoma. Other workers have found

ras gene mutations in between 39% and 47% of colorectal cancers.<sup>2, 29, 30</sup> Our results closely resemble those of Vogelstein *et al* with a higher prevalence of mutant K-ras genes in larger adenomas, adenomas harbouring cancer, and adenomas exhibiting severe dysplasia.

A particularly interesting finding was that of a benign tubulovillous polyp that contained both a mutant K-ras and a p53 allele. *In vitro* ras and p53 mutants cooperate to transform primary rat cells into cells capable of lethal tumourigenesis. This does not seem to be the case *in vivo*, at least not in the colorectum. Our findings suggest that this combination is insufficient on its own to cause an adenoma to become malignant. Supporting evidence for the non-cooperation of p53 and K-ras in colorectal tumourigenesis comes from our own finding and that of others that the frequency with which p53 and K-ras mutants are found together in large bowel cancers is only that to be expected by chance.<sup>31, 32</sup> If this combination of mutations were of biological significance preferential selection of cells with the double mutant phenotype might be expected, leading to clonal expansion of the mutant population. Another piece of evidence that p53 and K-ras do not cooperate in large bowel epithelium is the finding of ras mutation accompanied by p53 overexpression in an adenoma cell line, isolated by Dr C Paraskevas (personal communication). It may be that deletion of the remaining wild type p53 allele is also required before mutant p53 and K-ras exert their cooperative effect.

In conclusion, we have shown that p53 mutation, as indicated by overexpression of the protein and immunoreactivity with Pab 240, is an uncommon event in adenomas compared with colorectal carcinomas. K-ras codon 12 mutation, however, is much more common and seems to be related to size and degree of dysplasia. Unlike the situation *in vitro*, *in vivo* the combination of K-ras and p53 mutation seems to be insufficient to cause malignant change in a large bowel tumour. The critical event which determines malignancy in the adenoma-carcinoma sequence therefore remains unclear. Further definition of the *in vivo* interaction between p53 mutation, p53 deletion and K-ras mutation is urgently required.

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