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Letter to the Editor

Death pathway genes *Fas (Apo-1/CD95)* and *Bik (Nbk)* show no mutations in colorectal carcinomas

Dear Editor,

Many proteins with an established role in carcinogenesis and cancer progression (e.g. p53, RB, C-MYC, BCL2, RAS & E1B 19K) can regulate apoptosis and altered susceptibility to apoptosis may contribute to carcinogenesis.[1] Data from genetically manipulated animals show that oncosuppressor deficiency may abrogate the apoptosis of cells that have sustained DNA damage, thus permitting survival and proliferation of cells bearing mutations.[2] Apoptosis is widely observed in tumours, however, indicating that loss of ability to induce apoptosis – if important in carcinogenesis – must be restricted to particular pathways. Clear identification of these pathways in human tumours becomes an important aim, as it would help define the molecular basis not only of carcinogenesis but also of tumour resistance to various therapeutic measures. The study of colorectal cancers affords a particular advantage in this respect because a proportion have an underlying deficiency in DNA nucleotide mismatch repair (MMR), alternatively known as 'replication error positive' (RER+) phenotype. A high proportion of RER+ colorectal carcinomas do indeed bear clonally expanded mutations in microsatellite loci or repetitive sequences within cancer-related genes emphasising the importance of such genes in the process of carcinogenesis and cancer progression. Such mutated target genes include *APC*, *TGFβ-RII*,[3] and *Bax*,[4,5] all of which have also been implicated in pathways initiating apoptosis. Here, we provide information on two more death pathway genes: *Fas (Apo-1/CD95)* and *Bik-1 (Nbk)*.

Active FAS receptor triggers apoptosis in many cell types and appears to be necessary for apoptosis initiated by C-MYC and certain DNA-damaging chemotherapeutic agents. Binding of the FAS ligand induces trimerization of the receptor and this stimulates the intracytoplasmic FAS death domain to recruit a protein complex that includes caspase 8, thus activating the caspase cascade and hence the terminal effector events of apoptosis.[6] *Fas* is an attractive potential target for carcinogenic mutations. Individuals with the rare Canal-Smith syndrome, in which *Fas* is constitutively abnormal, have a high cancer incidence.[7] Moreover, FAS protein is constitutively expressed in normal colorectal epithelium and most colorectal adenomas, but expression is frequently less and sometimes undetectable in carcinomas.[8] The mechanism and significance of these changes, however, has not been established. BIK is a potent death-inducing protein sharing the BH3 domain of the BCL2 protein family. It has capacity to interact with the endogenous survival promoting proteins, BCL2 and BCL_{XL}, and their functional viral homologues BHRF1 and E1B-19K. BIK can induce apoptosis independently of p53 and BAX in some cell types[9] but its role in colorectal epithelium is unknown. We therefore studied the genes encoding FAS and BIK proteins in a series of primary colorectal carcinomas, to test the hypothesis that mutation in these apoptosis-promoting genes may be implicated in the process of human colorectal carcinogenesis.

DNA was extracted from frozen samples of 24 selected colorectal carcinomas (and normal mucosa) including 12 RER− and 12 RER+ after testing for microsatellite instability at 5–11 microsatellite loci (Figure 1a). Of the 12 RER+ tumours ten were right sided, ten had poor or mucinous differentiation, and six were immunohistochemically positive for p53 accumulation. The 12 RER tumours included three right-sided tumours, three with mucinous or poor differentiation and nine that were p53 positive by immunohistochemistry.

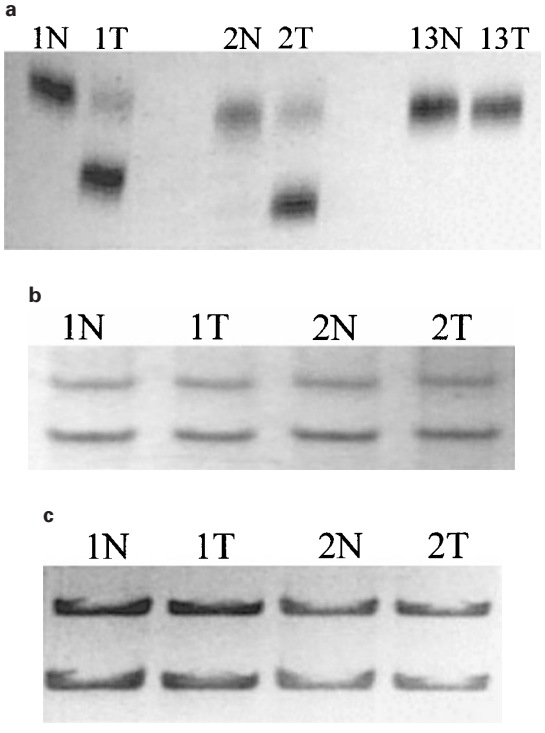

Figure 1 (**a**) Denaturing polyacrylamide gel showing RER characterisation at the BAT26 microsatellite locus: samples 1 and 2 show band shifts in the tumour lane (T) compared to the normal (N), consistent with derivation from RER+ cancers, whereas sample 13 does not show any band shifts and is derived from a RER− cancer. (**b**) SSCP for *Fas* exon 9I for samples 1 and 2 from RER+ tumours, showing no band shifts in the tumour (T) compared to the normal (N) lanes. (**c**) SSCP for *Bik* for samples 1 and 2 from RER+ tumours, showing no band shifts in the tumour (T) compared to the normal (N) lanes

Ten primer pairs were designed to PCR-amplify the nine *Fas* exons. *Bik* was examined less comprehensively, focusing on a potential target site for MMR deficiency mutations: a repetitive sequence containing CTG triplets [(CTG)2 GCG (CTG)5 GCG (CTG)3 CCG CTG] occupying positions 409–450, included within the Bik coding sequence. The PCR products were analyzed for mutations using single strand conformation polymorphism (SSCP).[10] Heteroduplex analysis[11] was performed on the PCR products from *Fas* exon 5 because these were difficult to resolve by SSCP. Where abnormal banding patterns were observed DNA was directly sequenced. By these criteria there was no evidence of mutation in any of the 24 cancer or normal mucosa samples examined, either in all the *Fas* gene exons (Figure 1b), or in the target repeat sequence in the *Bik* gene (Figure 1c).

On searching the coding sequence of *Fas* for nucleotide repeats we found a [(T)7] tract in exon 4, an [(A)6] tract in exon 9 and a [(T)5] tract in exon 3 in addition to some shorter repeat tracts. Other studies have shown that mononucleotide repeats of similar length are susceptible to MMR deficiency mutation and there is thus little doubt that *Fas* provides several intra-exonic targets for mutation in RER+ cells. The mutation screening method (SSCP) detects 80–90% of mutations when applied to single strand DNA between 150–250 nucleotides in length. We designed the PCR primers to generate products to fit this range as far as possible. Hence it is improbable that mutations in *Fas* or in the tandem repeat region identified in *Bik* are frequent in human colorectal cancers, even when these bear the mutation-susceptible RER+ phenotype.

The importance of clonal selection in carcinogenesis and tumour progression has recently been re-emphasised.[12] Our results contrast with the high incidence of mutation in RER+ colorectal cancers in other genes, notably *TGFβ-RII* (75%),[3] and *Bax* (50%).[4,5] This failure to identify any instance of clonal expansion of cells bearing *Fas* or *Bik* mutations thus suggests that such mutations confer no substantial growth advantage in colorectal carcinogenesis.

The question remains why FAS protein expression should be subnormal in a high proportion of colorectal carcinomas. The present results and those of others using Southern blot technology[13] exclude structural alterations in the gene. An obvious alternative mechanism would be depressed transcription secondary to reduced availability of wild type p53, since the first intron of *Fas* includes a p53 responsive element.[14] Of our 24 cases, 15 showed clear abnormality in p53, exhibited by immunohistochemical stabilisation affecting a high proportion of the nuclei. Although *Fas* down-regulation has the capacity to reduce cellular responses to lethal stimuli, it is significant that mutational inactivation of the gene is not observed in cancer tissues in which mutational events in other genes are common and clonally expanded. We conclude that *Fas* inactivation is unlikely to be a critical early event in colorectal carcinogenesis.

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