Mutations of Ki-ras and p53 genes in colorectal cancer and their prognostic significance

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Abstract

The series of genetic changes leading to malignancy in colorectal cancer is well reported. This includes mutational activation of the proto-oncogene Ki-ras and mutation/deletion of the p53 tumour suppressor gene. The frequency of these mutations was investigated in a panel of 52 colorectal cancer patients using a combination of immunocytochemistry and non-radioactive, digoxigenin-labelled *in situ* hybridisation. Sixty two per cent (32 of 52) of the study population were positive for p53 overexpression and 36% (19 of 52) positive for Ki-ras mutation. Twenty seven per cent (14 of 52) of the patients expressed both mutations. Mutation of either the p53 or the Ki-ras gene did not correlate with Dukes's stage, tumour differentiation or 5 year survival rate of the patients. Most of the rectal carcinoma specimens (11 of 15) showed p53 overexpression but the significance of this was not supported statistically. Thus detection of molecular changes is becoming more amenable to incorporation into routine histological carcinoma assessment because of the advent of non-radioactive labelling in *in situ* hybridisation and antibodies suitable for paraffin wax embedded specimens. The significance of these mutations in disease prognosis, however, remains questionable.

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Colorectal cancer is the result of an accumulation of genetic changes. These cancers are particularly well suited to studies of the progression of genetic changes leading to malignancy, as they tend to develop over years, if not decades. These acquired mutations affect fundamental cellular regulatory mechanisms, usually through the inactivation of tumour suppressor genes or the activation of oncogenes. These include the APC and MCC genes on chromosome 5q21,¹⁻³ the DCC gene on chromosome 18,⁴ the nm23 gene,⁵ the p53 gene on chromosome 17p,⁶ and the oncogenes Ki-ras and c-myc.^{7,8}

Evidence accrued to date shows that the p53 gene is the most commonly altered gene in human cancer, with mutations having been detected in breast,⁹ renal cell,¹⁰ oesophageal,¹¹ and non-small cell lung cancer.¹² It is a tumour suppressor gene, which in its normal form codes for a 53 kD protein, which binds to DNA and acts as a transcription factor to halt cells in the G1 to S transition in the cell

cycle.^{13,14} Mutant forms lack this DNA binding activity and thus cannot halt replication when DNA has become damaged and needs extra time for repair. This is the basis of Lane's functional model of p53 as a 'molecular policeman'.¹⁵

Deletions of chromosome 17p are found in 75% of colorectal cancers and are highly correlated with mutation of the remaining p53 gene.^{16,17} These mutations can be detected by immunocytochemistry, as the normally short lived protein becomes stabilised. Overexpression of p53 protein has been found in 42 to 67% of colorectal carcinomas^{18,19} and is generally considered to be one of the later genetic changes in the adenoma-carcinoma sequence.²⁰

The ras gene family consists of three closely related genes, H-ras, N-ras, and Ki-ras, which code for 21 kD proteins. These proteins are localised to the inner plasma membrane and can exist in two states: an active, signal transducing, GTP-binding state and an inactive, GDP-containing state.²¹ Mutations in Ki-ras are the ones most commonly associated with colorectal cancer,⁷ where they result in the loss of GTPase activity, causing the cell to be continuously activated and locked into proliferation. The incidence of mutated Ki-ras varies strongly among different tumour types, with only 4% of ovarian carcinomas showing an activated Ki-ras gene,²² whereas 42-50% of colorectal carcinomas had this mutation.^{20,23}

We wished to assess the relation between p53 and Ki-ras mutations and their prognostic significance in a series of colorectal cancer patients, using methods that could be incorporated into routine histological carcinoma assessment, namely immunocytochemistry and non-radioactive *in situ* hybridisation, and possibly enhance the accuracy of the currently used prognostic staging systems.

Methods

TUMOUR SAMPLES

Formalin fixed, paraffin wax embedded tissue sections from 52 colorectal cancer patients treated at Limerick Regional Hospital from 1987 to 1988 were used in the study. The study population consisted of four Dukes's A, 23 Dukes's B, 11 Dukes's C, and 14 Dukes's D patients. Of these tumours, 25 were left sided, 11 right sided, and 15 were rectal. When subdivided on the basis of differentiation, 27 of the tumours were well differentiated, 21 were moderately differentiated, and four were poorly differentiated.

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IMMUNOCYTOCHEMISTRY

Sections (5 μ m) were collected into poly-l-lysine-coated slides, dewaxed in xylene, and rehydrated through graded alcohols. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide solution (15 minutes). Normal rabbit serum at 1:5 dilution in TRIS buffered saline was used to block non-specific binding. Sections were incubated with primary antibody (DO-7 mouse monoclonal antibody, specific for human wild type and mutant p53 protein, Dako Ltd, High Wycombe, UK) at 1:100 dilution in TRIS buffered saline, overnight at 4°C. After incubation with a polyclonal biotinylated rabbit anti-mouse antibody (Dako Ltd, High Wycombe, UK) at 1:300 dilution in TRIS buffered saline for 40 minutes at room temperature, a StreptABComplex kit (Dako Ltd, High Wycombe, UK) with aminoethyl carbazole as chromogen was used to visualise the staining. Each section was incubated in the absence of primary antibody as a negative control. Tumours with strong p53 overexpression were used as positive controls.

NON-RADIOACTIVE IN SITU HYBRIDISATION

cDNA probe preparation

A cDNA probe, specific for the codon 12 mutation of the activated form of the human Ki-ras 2 oncogene²⁴ was isolated from clone ATCC 41027 according to standard techniques.²⁵ The random primer labelling system²⁶ was used to label the cDNA probe with digoxigenin-11-dUTP (Boehringer Mannheim, Lewes, UK). The prime-a-gene labelling kit (Promega Corp, Madison, WI, USA) was followed according to the manufacturer's instructions but was modified to permit incorporation of digoxigenin instead of ³²P – that is, the labelling mixture contained 1.5 mM each of unlabelled dATP, dCTP, dGTP, 0.97 mM dTTP, and 0.53 mM digoxigenin – 11-dUTP. The rest of the protocol remained unchanged.

Tissue preparation/hybridisation protocol

All of the normal precautions taken with in situ hybridisation to prevent RNAase or DNAase contamination were used throughout the procedure – that is, baking of glassware, use of sterile reagents/containers, incorporation of diethylpyrocarbonate in buffers, etc.

Tissue specimens of 5 μ m were collected onto poly-l-lysine coated slides, dried overnight at 37°C, dewaxed in xylene, and rehydrated through graded alcohols. After incubation in 10 mg/ml solution of proteinase K in TRIS buffered saline at 37°C, the slides were washed and then fixed in 4% paraformaldehyde solution in phosphate buffered saline at 4°C. The tissue was subjected to a prehybridisation step by incubating with the hybridisation cocktail, minus the cDNA probe, for one hour at 42°C. The cocktail consisted of 50% deionised formamide, 1×Denhardt's solution, 10% dextran sulphate, 10 mM dithiothreitol, and 18% TRIS-EDTA buffer (10 mM TRIS-HCl, 1 mM EDTA, pH 7.6). After addition of the digoxigenin-labelled cDNA probe, the sections were denatured at 98°C for 10 minutes, placed on ice for two minutes, and then incubated overnight at 42°C. Sections were washed next day in decreasing concentrations of SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.2) and the bound probe detected by immunocytochemistry using the digoxigenin nucleic acid detection kit (Boehringer Mannheim, Lewes, UK) with nitroblue tetrazolium chloride and bromochloroindolylphosphate for localisation of bound, alkaline phosphatase labelled antibody. Controls comprised of (a) pretreatment with 100 mg/ml RNAase A in 2×SSC/10 mM MgCl₂ for one hour at 37°C before hybridisation or (b) omission of labelled probe.

The efficacy of labelling was estimated by a dot blot technique where the response obtained from different concentrations of a labelled control DNA solution was compared with a dilution series of labelled cDNA probe. The response was detected using the reagents from the digoxigenin nucleic acid detection kit (Boehringer Mannheim).

STATISTICAL ANALYSIS

Frequency of p53 or Ki-ras positive tumours was compared for each variable using χ^2 statistics with the Solo Statistical System (BMDP Statistical Software, CA, USA).

Results

Overexpression of p53 protein was detected immunohistochemically in 32 of 52 (62%) of the tumour specimens examined. Staining was predominately nuclear and was rarely seen in adjacent normal mucosa (Fig 1). Activation of the Ki-ras gene was detected using a digoxigenin-labelled cDNA probe by non-radioactive in situ hybridisation in 19 of 52 (36%) of the study population. As the probe was specific for mRNA, specific staining was exclusively cytoplasmic in all tumour specimens examined (Fig 2).

Figure 1: Demonstration of nuclear p53 overexpression by immunocytochemistry in a colorectal tumour specimen.

Figure 2: Detection of cytoplasmic mutated Ki-ras oncogene by in situ hybridisation in a colorectal tumour specimen.

The relation between p53 or Ki-ras mutations and various clinicopathological variables was investigated (Table). Twenty five per cent of the Dukes's A specimens, 78% of the Dukes's B, 45% of the Dukes's C, and 50% of the Dukes's D specimens showed p53 overexpression. None of the Dukes's A specimens were positive for Ki-ras mutation whereas 35%, 54%, and 36% of the Dukes's B, C, and D specimens respectively, were positive.

Most of the rectal carcinomas showed p53 overexpression (73%), but the proportion of the left and right sided tumours that had p53 mutations was similar. Only 18% of the right sided tumours were positive for Ki-ras activation, with 33% of the rectal carcinomas and 52% of the left sided tumours showing Ki-ras mutations. Sixty three per cent of the well differentiated tumours were positive for p53 overexpression, with 57% of the moderately and 50% of the poorly differentiated tumours showing overexpression. None of the poorly differentiated, 29% of the well differentiated, and 53% of the moderately differentiated tumours showed Ki-ras mutations.

Of those patients whose tumours were positive for p53 overexpression, an equal number had died as had survived five years after their first presentation with disease (12 of 32, 38%). Similarly, there was no significant difference in the number of patients who had died and who had survived in the five years after their first

presentation with disease in the Ki-ras positive population (7 (35%) v 9 (45%) of 19). None of the clinicopathological variables were found to correlate with the expression of either p53 or Ki-ras mutations when analysed for statistical significance.

Discussion

Studies over the past five years have used various methods of detection to investigate p53 or Ki-ras mutations in colorectal cancer in an attempt to determine their prognostic significance. In a study using immunocytochemistry on frozen sections, Scott *et al*,¹⁹ reported that 22 of 52 (42%) of their samples were positive for p53, but no correlation was found with Dukes's stage, tumour grade or patient survival. When flow cytometry was used to quantify nuclear attached p53,¹⁸ 50 of 78 (64%) of the samples contained high levels of p53. These high levels correlated with tumour site, 17p allele loss, hyperdiploid DNA content, and also survival, but only when the Dukes's D samples were included in the analysis. Other studies using immunocytochemistry on paraffin wax embedded tissue also found a correlation between p53 content and survival,^{27 28} but differed in their assessment of significance of Dukes's staging. The use of different antibodies and tissue preparation procedures influences the extent of staining in these various studies, as the epitope on the p53 protein that a particular antibody is directed towards may not survive the fixation procedure. The frequency of antibody reactivity varied from 64 to 36% when three p53 antibodies were compared in a study on 90 frozen tissue specimens.²⁹ In general, however, there is quite good agreement between the different studies on the overall frequency of p53 overexpression, but to achieve statistical significance between this overexpression and some of the clinicopathological variables, a larger study population seems to be necessary. This is shown in our study, where the frequency of p53 positive tumours was similar to other reports – that is, 62% – but no correlation with Dukes's stage, tumour location, degree of differentiation or survival was established.

Mutations in the Ki-ras oncogene are thought to occur at an early stage in the adenoma-carcinoma sequence, with the frequency of mutations increasing with adenoma size.²⁰ In studies of Ki-ras mutation in gastrointestinal dysplasias, polymerase chain reaction amplification and sequence/restriction enzyme analysis of the mutated region seems to be the method of choice.^{23 30 31} This technique may not be available, however, in every laboratory and thus many have used specific antibodies to investigate levels of the ras gene product, p21, as a marker of mutational activation, with the monoclonal antibody Y13-259 being one of the most commonly used probes.³²⁻³⁴ We felt that the efficacy of this and other antibodies on formalin fixed, archival material was questionable and wanted to develop a technique that could possibly be

Clinicopathological variables and frequency of p53 overexpression/Ki-ras mutation

Variables	Cases (n)	No of p53 positive	No of Ki-ras positive (%)	
Dukes's stage				
A	4	1 (25)	0	NS*
B	23	18 (78)	8 (35)	NS
C	11	5 (45)	6 (54)	NS
D	14	7 (50)	5 (36)	NS
Location				
Right	11	6 (55)	2 (18)	NS
Left	25	13 (52)	13 (52)	NS
Rectum	15	11 (73)	5 (33)	NS
Differentiation				
Well	27	17 (63)	9 (33)	NS
Moderate	21	12 (57)	10 (48)	NS
Poor	4	2 (50)	0	NS

*NS=not significant, when tested using χ^2 analysis.

incorporated into routine histological carcinoma assessment, if it proved to be of prognostic significance. This is why an in situ hybridisation technique, using the non-radioactive label digoxigenin, was used to assess frequency of Ki-ras mutation. Thirty six per cent of the samples showed activation of the Ki-ras gene, but a correlation with either Dukes's stage, tumour differentiation, tumour location or survival was not found. In a study comparing ulcerative colitis associated and sporadic colorectal carcinomas, 14 of 33 (42%) of the sporadic cases were positive for Ki-ras codon 12 mutation, with a significant association with a rectal location.²³ Left sided tumour location is also frequently found to correlate with p53 overexpression,^{19 28} evidence that corroborates the theory of distinct patterns of tumour progression being associated with different sites in the colorectum.

Twenty seven per cent of the specimens studied here were positive for both p53 and Ki-ras mutations. The survival rate for patients with both mutations was not significantly different from those with only one or neither of the mutations (data not shown). This is in contrast with the study of 100 colorectal tumours by Bell *et al.*,³⁵ where the survival rate of patients expressing both mutations was nearly a third of that of the remainder of the series. It is not clear whether or not there is an association between the two types of mutation in tumour development. Enomoto *et al.*³⁶ report that ras and p53 gene mutations occur independently in endometrial adenocarcinoma. This was also seen in non-small cell lung cancer.³⁷ In a mouse prostate model, the expression of activated ras and myc oncogenes induced carcinoma development but bypassed the need for p53 mutation by neutralising the tumour suppressor activity of normal p53.³⁸ Cooperation between ras cDNA and mutant p53 was required, however, to fully transform rat embryo fibroblasts, as neither cDNA alone accomplished this to the same extent.³⁹ To add further confusion, the presence of both p53 overexpression and Ki-ras mutation was not sufficient to cause transformation of benign large bowel adenomas in two separate studies of these mutations in colorectal adenomas.^{40 41} It would seem that the in vivo situation and that seen in model systems differ quite significantly. Many factors other than mutations in p53 and Ki-ras genes determine the progression of disease (for example, mutations in the DCC, APC or nm23 genes, environmental factors, etc), thereby making the assessment of prognostic significance of the p53 and Ki-ras mutations difficult. These other factors may be partially responsible for the differing survival rates reported for patients expressing both mutations (for example,³⁵ this study). In our study there was no evidence to suggest that the presence of either or both mutations was prognostically less favourable or that either one occurs earlier or later in the progression of disease. Thus, even though techniques are now available that could be incorporated into routine histological carcinoma assessment,

namely immunocytochemistry on paraffin wax embedded tissue and non-radioactive in situ hybridisation, the prognostic significance of these mutations remains questionable.

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