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Original Paper

The Candidate Tumour Suppressor Gene, *ING1*, is Retained in Colorectal Carcinomas

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***ING1* plays a critical role in regulating cell cycle progression and susceptibility to apoptosis. The present study aimed to investigate allelic deletion of, and mutations within, the *ING1* gene in colorectal carcinomas. Genomic DNA was extracted from 29 sporadic colorectal carcinomas and samples of adjacent normal mucosa. Losses of heterozygosity of two polymorphic dinucleotide repeat markers close to the *ING1* locus at chromosome 13q32-34 were analysed. Single-stranded conformational polymorphisms of polymerase chain reaction amplified regions within the coding sequence of *ING1* were examined. Microsatellite instability was noted in 5 (17%) colorectal carcinomas; this confirms selection of a subject sample representative of the population. Neither losses of heterozygosity nor changes in electrophoretic mobility of single-stranded polymerase chain reaction products were detected in any colorectal carcinoma. Thus, in common with tumour suppressor genes such as *RB* and *BRCA2* on chromosome 13q, *ING1* appears to be retained intact in colorectal carcinomas.**

Key words: chromosome deletion, colorectal neoplasms, gene deletion, loss of heterozygosity, mutation, polymerase chain reaction, polymorphism, single-stranded conformational
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INTRODUCTION

Loss of function of the *TP53* tumour suppressor gene is an important event in the pathways leading to unregulated epithelial cell proliferation during development and progression of several human cancers [1]. Anti-proliferative activities of p53 have recently been shown to depend on the intact functioning of another candidate tumour suppressor gene, designated as *ING1*, which was identified by a cloning strategy aimed at genes whose expression is selectively reduced in cancer cells [2]. Over-expression of *ING1* has been found to block cell proliferation [2] and increase programmed cell death in the absence of survival factors [3]. When expression of *ING1* is reduced using antisense mRNA, unrestrained proliferation and illegitimate cell survival are promoted [3]. However, $p33^{ING1}$, the protein encoded by this gene, can neither block proliferation of cells lacking wild-type p53 nor render p53-deficient cells sensitive to chemotherapy [4]. Conversely, if production of $p33^{ING1}$ is suppressed using antisense RNA, cells can escape wild-type p53 mediated growth inhibition. The interaction of $p33^{ING1}$ and p53 to form a specific protein–protein complex appears to increase the efficiency of p53 as a transcriptional activator [4].

The *ING1* gene has been mapped to chromosome 13q33–34 by fluorescence *in situ* hybridisation [5]. Losses of heterozygosity at sites close to this locus on chromosome 13q have been reported in gastric adenocarcinomas [6] as well as head and neck squamous cell carcinomas [7]. These reports suggest allelic deletion of an as yet unidentified tumour suppressor gene for which *ING1* now emerges as a prime candidate. The possibility of mutational inactivation of the retained allele of this gene is suggested by limited studies in a transformed cell line [2]. In view of these data, the need for detailed assessment of alterations in the *ING1* gene in human cancers, particularly those in which loss of wild-type p53 is a common event, has been recently emphasised [8]. From the

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perspective of understanding the genetic changes that underlie the development of malignancy, colorectal carcinoma is one of the most fully characterised tumour systems. Consequently, the present study aimed to investigate allelic deletion of, and mutations within, the *ING1* gene in colorectal cancers at different stages.

PATIENTS AND METHODS

Tissue samples

Biopsies of primary colorectal carcinomas and normal mucosa from the margin of resected specimens were obtained from patients undergoing operation. All biopsies were immediately embedded in tissue-freezing medium in liquid nitrogen and cryosections were stained with haematoxylin and eosin. Immediately consecutive tissue sections were collected and stored at $-80°C$. Sections from 29 tumour biopsies, each containing more than 70% malignant cells, and paired normal mucosal sections were included in this study. Microdissection of tumour specimens was not attempted. Pathological staging of cancers was conducted according to the TNM system and included stage I ($n=5$), stage II ($n=10$), stage III ($n=9$) and stage IV ($n=5$) carcinomas. These studies were approved by the institutional ethics committees of St James's and Seacroft University Hospitals NHS Trust.

Genomic DNA (gDNA) extraction

Extraction was conducted using the QIAamp tissue kit (Qiagen, Crawley, U.K.), as per the manufacturer's protocol. Collected cryostat sections weighing approximately 25 mg were incubated with a tissue lysis buffer and proteinase K (Sigma, Dorset, U.K.) at 55°C, until the tissue was completely lysed. gDNA was adsorbed on to the silica membranes in spin columns, eluted in a subsequent step and quantified by spectrophotometry.

Loss of heterozygosity (LOH) analyses

Oligonucleotide primers specific for two highly polymorphic dinucleotide repeat markers located on chromosome 13q close to the *ING1* gene locus were synthesised. These were D13S129 (13q32) dACC AAA ACT GCA CAA ATG CCA and D13S174 (13q32-33) dGAC GAC TAA CCT CAA GTG CG (forward) and dTGA AGG CAG AAG TAA AAC CAT TAT C (reverse) [9]. Polymerase chain reaction (PCR) was carried out in a final volume of 20 µl containing 200 ng gDNA, 20 pmol of each oligonucleotide primer, 2 mM magnesium chloride (Promega, St Albans, U.K.), 0.2 U *Taq* DNA polymerase (Promega), 0.2 mM of each dNTP (Promega), magnesium-free buffer (Promega) and 0.2 Mbq [α-^{33}P]dATP (Amersham, St Albans, U.K.). Twenty-five cycles of PCR amplification were performed in a DNA thermal cycler (MJ Research Inc, Essex, U.K.) with denaturing at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. The final extension step was conducted at 72°C for 5 min. PCR products were mixed with an equal volume of denaturing solution (23.75 ml 99% formamide, 1.25 ml 1% xylene cyanol and 10 mg bromophenol blue in 25 ml), heated at 95°C for 5 min and quenched on ice to prevent re-annealing of single-stranded products. Normal and tumour samples were loaded in adjacent lanes of a 6% denaturing polyacrylamide gel (Sequagel-6, National Diagnostics, Hull, U.K.) and subjected to electrophoretic separation and autoradiography. It was decided to score LOH as present if the signal intensity was reduced to 50% or less.

Polymerase chain reaction—single stranded conformational polymorphism (PCR–SSCP) analyses

Four pairs of oligonucleotide primers were designed to span the coding sequence of *ING1* (Accession number: AF001954). These were: AA′, d GAT CCT GAA GGA GCT AGA CGA (forward; positions 198–218) and dCAC CAT CTG GCT CAC GAT CT (reverse; positions 329–348); BB′, dGAA GAT CCA GAT CGT GAG CC (forward; positions 305–324) and dGCT GTT GGG CTT GTC AGA CT (reverse; positions 466–485); CC′, dAGT CTG ACA AGC CCA ACA GC (forward; positions 466–485) and dGGT TCG TTG GGG TCG ATG (reverse; positions 673–656); and DD′, dACG AAC CCA CGT ACT GTC T (forward; positions 667–685) and dTAC CTG TTG TAA GCC CTC TC (reverse; positions 864–883). A BLAST search was performed to confirm that these primers were apparently specific for *ING1*. PCR was carried out in a final volume of 25 µl containing 200 ng gDNA, 20 pmol of each oligonucleotide primer, 2 mM magnesium chloride, 0.625 U *Taq* DNA polymerase, 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl and 0.2 mM of each dNTP in pre-aliquoted tubes (Advanced Biotechnologies, Epsom, U.K.). PCR kinetics and denaturing of samples were as described in the preceding section. Normal and tumour samples (6 µl each) were loaded in adjacent lanes of a 12.5% polyacrylamide gel (GeneGel Excel, Pharmacia Biotech, St Albans, U.K.), subjected to electrophoresis (GenePhor unit, Pharmacia Biotech) and DNA fragments visualised by silver staining (PlusOne DNA silver staining kit, Pharmacia Biotech), according to the manufacturer's protocols.

RESULTS

LOH analyses

Microsatellite instability was detected in five (17%) cancers (Figure 1) and these were excluded from LOH analyses because allelic loss patterns in tumours showing microsatellite instability are difficult to interpret.The marker D13S129 was informative (i.e. heterozygous) in 23 of the 29 pairs of samples and D13S174 was informative in 27 sample pairs. LOH of either of these markers was not detected in any tumour.

PCR–SSCP analyses

Mutations in the coding sequence of *ING1* were investigated by SSCP analyses using four different primer pairs. No electrophoretic band shifts were detected in any of the 29 cancer specimens, compared with matched normal colorectal mucosa (Figure 2).

DISCUSSION

Allelic losses are hallmarks of chromosomal regions harbouring tumour suppressor genes. In sporadic cancers, inactivation of both tumour suppressor alleles occurs somatically and LOH is presumed to provide a growth advantage to the tumour cell because a prior somatic mutation has already inactivated one allele [10]. For example, tumour suppressor genes such as *APC* and *TP53*, which have been implicated in the pathogenesis of colorectal carcinoma, are located at sites on chromosomes 5q and 17p, respectively, which frequently show regions of deletion in colorectal carcinomas [11]. In contrast, LOH at sites on chromosome 13 which bear the *RB* and *BRCA2* tumour suppressor genes has not been reported in colorectal carcinomas, although inactivation of these genes

is known to be critical in the pathogenesis of other human cancers [12]. Similarly, the present study demonstrates that, although loss of alleles at distal chromosome 13q has been reported in gastric [6] as well as head and neck cancers [7], it does not appear to occur in colorectal carcinomas. It is unlikely that contamination with excessive normal tissue resulted in this failure to detect allelic loss because microsatellite instability was detected in a proportion of cancers, consistent with other studies [13, 14]. The detection of either microsatellite instability or LOH demonstrates the presence of a clonal population of cells that share altered genetic information, which is a characteristic of cancer. The present results, therefore, imply that allelic deletion is not a mechanism for inactivation of *ING1* if, indeed, this gene is involved in colorectal carcinogenesis.

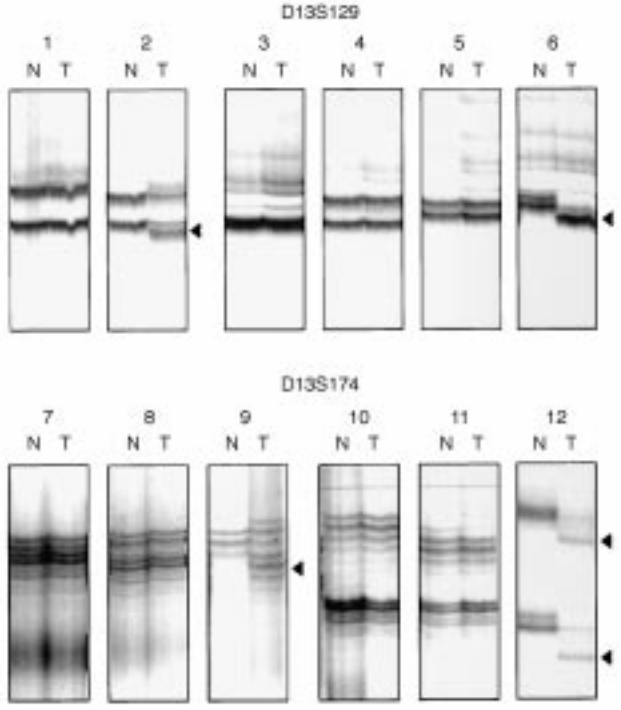

Figure 1. Loss of heterozygosity analyses at 13q showing paired normal (N) and colorectal carcinoma (T) samples. For D13S129, cases 1, 4, and 5 were informative with no LOH, case 3 was non-informative and cases 2 and 6 show microsatellite instability (indicated by arrowheads). With D13S174, cases 8, 10 and 11 are informative with no LOH, case 7 is non-informative and cases 9 and 12 show microsatellite instability.

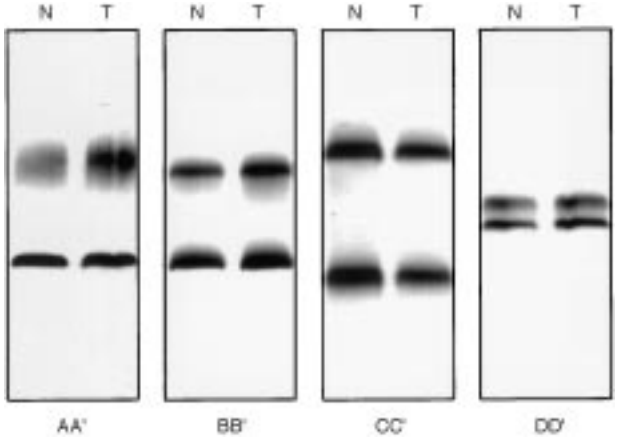

Figure 2. Representative results of polymerase chain reaction—single stranded conformational polymorphism analyses using four different primer pairs designated as AA′, BB′, CC′ and DD′. Paired lanes are normal (N) and colorectal carcinoma (T) samples from the same patient. No shifts in electrophoretic mobility are noted.

It was also considered possible that *ING1* may not behave as a classical tumour suppressor gene, which acts in a recessive manner at the cellular level and requires inactivation of both alleles for full deregulation to occur. Consequently, the presence of mutations within the sequence of *ING1* was examined by SSCP analyses. Changes in the electrophoretic mobility of DNA fragments, indicative of mutations, were not detected in any of the cancer samples. These results imply that mutations in *ING1* are not a common feature of colorectal carcinomas.

In conclusion, allelic deletion of, or mutations within, the *ING1* gene do not appear to occur during colorectal carcinogenesis. Absence of either of these alterations suggests that *ING1* does not behave like a classical tumour suppressor gene in the pathogenesis of human colorectal carcinoma. However, it is possible that an alternative mechanism for loss of gene expression may be operational. For example, hypermethylation of the gene promoter site is responsible for inactivation of tumour suppressor genes such as *p16(INK4)* [15], *E-cadherin* [16] and *BRCA1* [17]. Hypermethylation has recently been implicated as one mechanism for age-associated silencing of tumour suppressor genes in sporadic colorectal carcinomas [18] and further studies are required to clarify whether *ING1* expression is altered by such a process.

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