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ORIGINAL ARTICLE

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Tumour-derived mutated K-*ras* codon 12 expression in regional lymph nodes of stage II colorectal cancer patients is not associated with increased risk of cancer-related death

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Abstract This study examined the frequency of lymph node micrometastases detected by expression of mutant K-*ras* oncogene present in the respective primary tumour. The study population consisted of consecutive patients with stage II colorectal cancer (CRC) undergoing curative resection and with disease-free survival of 60 months or longer or CRC-related death. Of 27 patients found to have K-*ras* mutations at codon 12, 17 had genomic DNA suitable for PCR recovered from corresponding regional lymph node tissue. The same K-*ras* mutation was identified in the lymph nodes of 13 patients (76%), four of whom (30%) died of CRC recurrence within 5 years. A single patient in the negative group (25%) also died. Lymph node micrometastases detected by this technique thus show no relationship to mortality in stage II CRC. Further study of this technique is necessary before it can be used in the selection of patients for adjuvant chemotherapy

Keywords Colorectal cancer · Survival · Micrometastases · k-*ras* · Cytokeratins

Introduction

Each year 500,000 persons are estimated to die of colorectal cancer (CRC). The incidence is particularly high in Western industrialized nations. CRC is a very significant public health issue in Ireland [1], which has the third and fifth highest CRC mortality for men and women, respectively. Population screening can reduce CRC mortality [2] but is a costly strategy [3], and resources are not currently available to implement such a programme in Ireland. Given these circumstances, improving the outcome of CRC with better chemotherapy and optimized patient selection may offer a more affordable means of reducing overall CRC mortality. Adjuvant chemotherapy is now routinely offered to patients with stage III (Dukes' C) CRC and to a subgroup of patients with node-negative rectal tumours with definite survival advantage. Unfortunately, adjuvant therapy has not been shown unequivocally to benefit those with non-rectal stage II CRC [4].

In stage II patients there are two distinct patterns of survival which existing staging methods are incapable of distinguishing prospectively [5]. A key issue is the determination of prognostic markers that are able to identify subgroups of apparently early-stage patients at high risk of disease relapse who may also benefit from adjuvant therapy. In the absence of established metastatic disease, regional lymph node involvement is the most important independent prognostic factor [6]. By inference, strategies aimed at augmenting the detection of lymph node metastases would likely improve current staging for stage II patients.

Micrometastases have previously been identified using keratin immunohistochemistry. This technique was

first described for CRC in 1990 by Davidson et al. [7]. Series based on this method have shown contradictory results in terms of the relationship between survival and detection of micrometastases [7, 8, 9, 10, 11, 12, 13]. No consensus exists as to their relevance to survival in these patients and the existing data are somewhat confusing and mutually contradictory. Immunohistochemistry is a relatively insensitive technique. Molecular biological techniques can offer greater sensitivity. Small studies have also demonstrated lymph node expression of carcinoembryonic antigen and keratin filament RNA with mortality in a variety of gastrointestinal malignancies [14] and specifically in stage II CRC [15, 16]. Hayashi et al. [17] discovered a statistically significant association between cancer-related mortality and lymph node expression of mutant K-*ras* alleles detected in primary tumours using a mutant allele specific amplification polymerase chain reaction (PCR) technique in a cohort of Japanese patients.

K-*ras* is located on the short arm of chromosome 12 (12p12.1). It belongs to the supra-family of *ras* oncogenes which also includes H-*ras* and N-*ras*. These are located on different chromosomes, but their products are homologous. K-*ras* gene consists of four exons, of which the fourth may be alternatively spliced. It encodes a 21-kDa G protein which contains either 188 or 189 peptides depending on the splicing of the fourth exon. It is involved in the transduction of signals stimulating cell division across the cell membrane [18].

Codon mutations have been identified in 39–71% of CRC cases [18, 19, 20, 21, 22]. Codon 12 is most frequently mutated in CRC. It is a mis-sense mutation, allowing glycine to be substituted for another amino acid. Mitogenesis is activated when RAS binds GTP; the wild-type protein then hydrolyses the bound GTP and results in the inactive complex of RAS-GDP. The mutant gene product is thought to be unable to hydrolyse GTP resulting in a continuous stimulus to divide [22].

We investigated the frequency of lymph node micrometastases as detected by PCR for K-*ras* codon 12 mutations in a cohort of Irish patients with stage II CRC.

Patients and methods

The initial study population comprised 27 patients who underwent curative resections for CRC between 1985 and 1991 and have been followed up to study end points defined as either survival for at least 5 years or a CRC-related death within 5 years of diagnosis, and whose archived tissue yielded genomic DNA suitable for PCR. All subjects had CRC of stage II, with haematoxylin and eosin stained nodal histopathology reported by a consultant histopathologist. Archived paraffin-fixed tumour and lymph node tissue was retrieved in each patient. Survival data were obtained either from hospital records or from primary care physicians, medical insurance companies or next of kin.

DNA extraction

All solvents were obtained from BDH (Poole, UK). Approximately 30 5-μm sections of each tumour and lymph node block were cut on a microtome. Sections were de-paraffinized by homogenization with xylene; the DNA-containing fraction was extracted to absolute ethanol and digested with proteinase K sodium dodecyl sulfate buffer (Sigma, St. Louis, Mo., USA). DNA was then extracted using a standard phenol-chloroform technique. DNA was precipitated in absolute ethanol and reconstituted in approx. 200–600 μl water depending on DNA concentration determined by spectrophotometry (Beckman Instruments, Fullerton, Calif., USA).

PCR conditions

Primer sequences 5′ TAAACTTGTGTGGTAGTTGGAGCC 3′ and 5′TCTATTGTTGGATCATATTC 3′ were synthesized. From each extracted DNA sample 5 ml was added to 90 μl master mix solution comprising; 63 μl sterile water, 10 μl stock magnesium chloride (supplied with *Taq*), 10 μl *Taq* buffer, 1 μl dNTP solution (×4), 1 μl primer solution (×2) and 1 μl *Taq* enzyme (Promega, Madison, Wis., USA.) The following protocol was employed; denaturation, 35 cycles of 1 min at 94°; annealing, 35 cycles of 30 s at 54°; extension, 35 cycles of 1 min at 72°. The products were subjected to electrophoresis on 4% Nu-Sieve/TAE gel (FMC, Rockland, Me., USA) using ethidium bromide and ultra-violet transillumination.

Successful PCR was indicated by the presence of a 99 bp band.

Enzyme restriction

From the PCR product 40 μl was digested with 2 μl buffer and 1 μl *Msp*I restriction enzyme (Promega) for 16 h at 37°C. The restriction reaction was terminated by standing solutions at –20°C for 20 min. The reaction products were allowed to stand at room temperature in order to thaw. The products were subjected to electrophoresis on 4% Nu-Sieve/TAE gel (FMC) using ethidium bromide and ultra-violet transillumination. A codon 12 mutation was indicated by detection of fragments of both 99 and 78 bp [21].

Results

K-*ras* mutations at codon 12 were found in 27 subjects. In 17 of these the regional lymph nodes yielded DNA suitable for PCR, and 13 of these (76%) had the same mutations at codon 12 while 4 (24%) did not (Figs. 1, 2). Of the positive group 4 (31%) died of CRC within 60 months of resection while a single patient (25%) died in the negative group (Table 1). The 5-year survival rate was 69% in the positive group and 75% in the negative group. To clarify the patho-physiological mechanism underlying nodal K-*ras* expression we stained corresponding 7-μm lymph node sections with a monoclonal antibody raised against cytokeratins 5, 6, 8 and 17 using Dako Antibody M0821 (Dako, Copenhagen). The regional lymph nodes of 25 subjects had either single or clustered cells that stained positive for cytokeratins. We examined the association between nodal mutant K-*ras* codon 12 expression and cytokeratin detection using Kendall's τ_b coefficient. No association was found (P=0.151; Table 2).

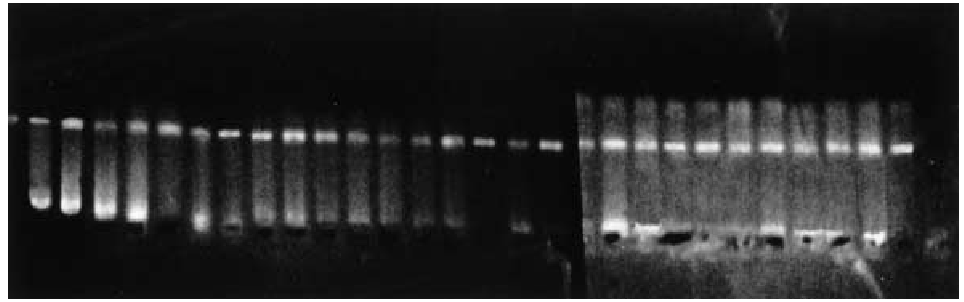

Fig. 1 Is a composite photograph of a gel transilluminated with UV light. Fragments of 99 bp are shown, indicating successful PCR for K-*ras* codon 12

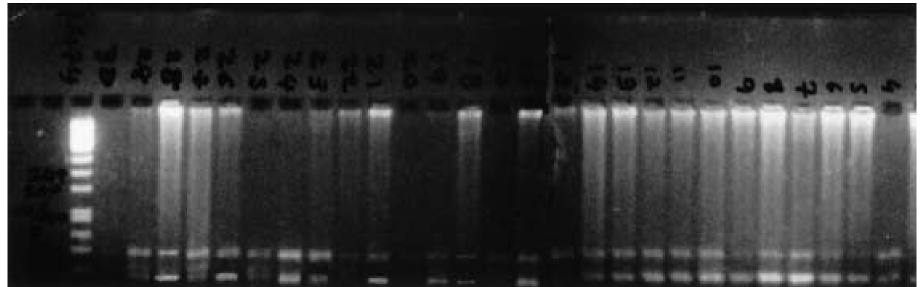

Fig. 2 Is a composite photograph of a gel transilluminated with UV light. A codon 12 mutation is indicated by the presence of both 99- and 78-bp bands

Table 1 Outcome versus nodal K-*ras* detection

	CRC death	Survival	Total
Nodal negative	1	3	4
Mutant K-*ras* positive	4	9	13
Total	5	12	17

Table 2 Nodal mutant K-*ras* versus cytokeratin detection

	Nodal negative	Cytokeratin positive	Total
Nodal negative	1	3	4
Mutant K-*ras* positive	6	7	13
Total	7	10	17

Discussion

The detection of K-*ras* mutations by PCR is an ideal surrogate for occult lymph node disease because of several factors. No study pertaining to CRC has found a definitive association between reduced survival and primary tumour K-*ras* mutations, They are thought to be among the earliest events in colorectal tumorigenesis and are uniquely stable. Losi et al. [20] reported a series of 32 colorectal cancer patients, 25 of whom had c-K-*ras* mutations, with 23 at codon 12. They also observed this mutation in associated distal metastases and found that no tumour metastases expressed c-K-*ras* mutations if not present in the respective primary tumour.

A major disadvantage associated with employing PCR for K-*ras* codon 12 as a means of detecting micrometastases is that not all paraffin-embedded, formalin-fixed tissue yields genomic DNA suitable for PCR analysis. It is necessary to fix and paraffin embed tissue to facilitate conventional histopathological diagnosis (haematoxylin and eosin staining). An alternative would be to remove a small sample from fresh lymph node tissue, assuming it could be easily identified within the resected mesenteric tissue; However, this would introduce sampling bias, might compromise conventional histopathology and would require a prospective follow study, which would not yield definitive results until at least 60 months had passed from its inception.

Once this obstacle is overcome, further difficulties are engendered because all tumours do not express K-*ras* mutants. The reported incidence of *ras* mutations ranges in various series from a minimum of 39% to a maximum of 75%. Of those expressing K-*ras* mutants approx. 90% do so at codon 12 [18, 19, 20]. Given the expected frequency of K-*ras* mutations in CRC and the limitations of DNA extraction from paraffin tissue, the best overall yield that can reasonably be expected is in the order of 40% (slightly less if only codon 12 is sought). Therefore strategies aimed at detecting lymph node micrometastases using PCR for c-K-*ras* are quite limited and not suitable as stand-alone measures.

In general the advantages of PCR analysis include unparalleled sensitivity, unequivocal objectivity of inter-

pretation and reproducibility. Its sensitivity exposes the method to a certain risk of false-positive results, mainly from cross-contamination, which can be avoided through meticulous technique. The detection of mutant K-*ras* within lymph node tissue does not in itself imply that viable tumour material is present. Both the present study and that of Lieffers et al. [16] found a frequency of occult lymph node disease that far exceeds that of cancer-related death in stage II patients. The lack of correlation with detection of keratin-positive epithelial cells is troublesome and difficult to account for. We speculate that non-viable remnants of tumour cell nuclei that have been destroyed by the host immune response or have undergone apoptosis are transported to regional lymph nodes, thus accounting for the high frequency of "micrometastases" detected by PCR techniques.

In summary, three-quarters of stage II CRC patients were found to have K-*ras* codon 12 mutations in both lymph nodes and primary tumours. This detection was not correlated with cancer death or with immunohistochemical detection of keratins within lymph nodes. Further study is necessary before such findings can be assigned a prognostic value or used as a means of selecting patients for adjuvant therapy.

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