

ORIGINAL ARTICLE

Ki-ras gene mutations, LOH of the APC and DCC genes, and microsatellite instability in primary colorectal carcinoma are not associated with micrometastases in pericolic lymph nodes or with patients' survival

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Aims: The primary aim of this study was to look for possible correlations between molecular genetic changes in primary colorectal cancer and the presence or absence of micrometastases in the accompanying pericolic lymph nodes. The secondary aim was to correlate the data on these molecular genetic changes and micrometastases with survival.

Methods: One hundred and twenty five Dukes's stage B colorectal cancers from 1989 to 1992 were analysed. The primary tumours were evaluated for Ki-ras mutation, adenomatous polyposis coli (APC) loss of heterozygosity (LOH), deleted in colon cancer (DCC) LOH, and microsatellite instability using standard molecular techniques. All available lymph nodes were immunohistochemically stained for micrometastases.

Results: Micrometastases were present in 41% of patients. There were significantly more lymph nodes removed in the patients with micrometastases. Micrometastases were not associated with Ki-ras mutation, APC LOH, DCC LOH, or microsatellite instability, even when controlling for the number of lymph nodes removed. None of the molecular variables considered had a significant impact on either overall survival or on death with disease.

Conclusions: There are insufficient data to justify using molecular genetic changes in primary colorectal carcinomas as prognostic markers. Micrometastases do not provide prognostic information on survival. There is value in increasing the numbers of lymph nodes removed and analysed along with the primary tumour.

Colon cancer is the second most common cause of cancer death in the USA, with approximately 150 000 new cases diagnosed each year. Tumour staging, as a reflection of the extent of disease, is well established as the single most important factor in determining the risk of recurrence and prognosis for survival.¹ The five year survival of patients with stage I disease is greater than 80%, but only 70% or less for those patients with stage II disease receiving surgery only.² Stage III disease has been shown to benefit from adjuvant chemotherapy after surgical removal.³ Because the tumour will recur in 30% of patients with stage II disease, it is reasonable to assume that these patients might benefit from adjuvant chemotherapy, similar to those with stage III disease. It has been difficult to identify those patients with stage II disease who are destined to relapse, thereby avoiding treatment for those with a more favourable outcome. Various clinical, pathological, and molecular features have been evaluated in this regard, including molecular genetic changes in specific genes, microsatellite instability (MSI), and the presence of micrometastases.⁴ In particular, it would seem plausible that the presence of micrometastases, or limited tumour cells, within pericolic lymph nodes would be a precursor for subsequent tumour spread. However, to date, none of these variables has been identified as a consistent predictor.

"It has been difficult to identify those patients with stage II colorectal cancer who are destined to relapse, thereby avoiding treatment for those with a more favourable outcome"

The primary goal of our study was to look for possible correlations between certain molecular genetic changes in primary colorectal cancer and the presence or absence of micrometastases in the accompanying pericolic lymph nodes. Our secondary aim was to correlate the data on these molecular genetic changes and micrometastases with survival.

MATERIALS AND METHODS

Patient material

We identified 150 patients with Dukes's stage B colorectal cancer treated at our hospital with primary surgery from 1989 to 1992, none of whom received adjuvant treatment. However, only 125 patients were studied because paraffin wax blocks of lymph nodes or primary tumours were not available for 25. The tumour registry provided survival information. Our study was approved by the hospital institutional review board. All data were computerised anonymously.

DNA extraction and purification

All tissue samples were formalin fixed and paraffin wax embedded. Several sections of each were cut on to slides and one of the sections was stained with haematoxylin and eosin (H&E) to identify neoplastic tissue. The histologically relevant regions from the unstained sections were isolated

Abbreviations: APC, adenomatous polyposis coli; DCC, deleted in colon cancer; H&E, haematoxylin and eosin; LOH, loss of heterozygosity; MSI, microsatellite instability; PCR, polymerase chain reaction; SSCP, single stranded conformation polymorphism

using a blade and transferred to an Eppendorf tube. The paraffin wax was removed by xylene and ethanol washes. The cellular material was lysed in a proteinase K buffer solution. DNA was isolated and purified using the QIAmp DNA mini kit (Qiagen Inc, Valencia, California, USA). Genomic DNA was stored at 4°C in 10mM Tris EDTA buffer (pH 9.0).

LOH analysis of the APC and DCC genes

Loss of heterozygosity (LOH) of the adenomatous polyposis coli (APC) gene was determined through the polymerase chain reaction (PCR) amplification of a CA repeat microsatellite marker within the D5S346 locus of the DP1 gene.⁵ Samples that were homozygous for the D5S346 primer set were analysed using primer sets for CA repeats within the D5S1965 and/or D5S492 loci.⁶ LOH of the deleted in colon cancer (DCC) gene was determined by amplification of the CA repeat markers within the D18S58 or D18S61 loci.⁷

PCR reactions were carried out in 30 µl volumes using reagents from Applied Biosystems (Roche Molecular Systems Inc, Branchburg, New Jersey, USA). Four picomoles of each primer and a 1.5mM MgCl₂ concentration were used in the PCR reactions. All PCR primer sets for microsatellite analysis had a 5'-fluorescence label on the sense strand and a 5'GTGTCTT tail on the antisense strand. Reactions were run on a PE 9700 thermocycler (PE Applied Biosystems, Foster City, California, USA), as reported previously.⁸ Normal colonic mucosal tissue from the same patient was evaluated at the same time as the neoplastic tissue for all LOH studies. LOH was defined as reported previously.⁹

MSI analysis

The mononucleotide repeat BAT26 was used to screen for MSI in neoplastic tissue specimens using the same conditions as for the microsatellites in the LOH analyses.¹⁰ BAT26 has been shown to identify neoplasms with a high degree of MSI.¹¹ However, the D5S346 and D18S58 (or D18S61) dinucleotide repeats are sensitive to MSI and the data collected for the LOH analyses were used along with the BAT26 data as an MSI panel of at least three markers. Neoplastic tissue showing bands that were not present in the corresponding normal tissue was classified as MSI positive.

Mutation analysis of the Ki-ras gene

Single stranded conformation polymorphism (SSCP) analysis was used to screen for mutations within the codon 12/13 region of exon 1 of the Ki-ras oncogene.¹² The sense and antisense primers were 5'-labelled with different fluorescent tags. PCR reactions were carried out in 30 µl volumes using

reagents from Applied Biosystems. The samples were analysed on an ABI Prism 377 DNA sequencer with GeneScan collection software (PE Applied Biosystems). Normal samples were run alongside neoplastic specimens to identify the wild-type SSCP banding pattern. Each sample that produced a mutant SSCP band was sequenced to verify the point mutation. PCR based DyeDeoxy Terminator sequencing was performed using an ABI Prism 377 DNA Sequencing System (PE Biosystems).

Immunohistochemical analysis

Four 5 µm thick sections of formalin fixed, paraffin wax embedded pericolic lymph nodes were cut, mounted on Fisher SuperFrost Plus slides, dewaxed, and pretreated with pepsin. Monoclonal antibodies to keratin AE1/AE3 and keratin CAM 5.2 were applied to the sections. The slides were incubated to allow binding of the antibody to the antigen, followed by a detector system, and counterstained with haematoxylin. A single cell or clusters of cells in lymph nodes that stained with either the antikeratin AE1/AE3 or antikeratin CAM 5.2 antibody indicated the presence of micrometastases from the colorectal cancer. Positive and negative controls accompanied each test run (fig 1).

STATISTICAL METHODS

The χ^2 statistic was used to assess the association between the markers and micrometastasis status. The relative risk and 95% confidence interval were derived to assess whether the markers were risk factors for micrometastasis. The Mann-Whitney U test was used to compare numbers of lymph nodes according to micrometastasis status. Kaplan-Meier curves of survival were derived for each marker. The log rank statistic was used to compare patient survival according to marker status. The Cox proportional hazards model with Breslow's approximation method for ties was used to assess whether various markers were independent predictors for survival, and the effect of age.

RESULTS

Of the 125 patients, 52% were female and 48% were male. The mean ages of the women and men were 73 and 69 years, respectively. Only nine individuals were under the age of 50 years. The carcinomas had the following distribution: caecum, 31 (24.8%); ascending colon, 19 (15.2%); transverse colon, 12 (9.6%); descending colon, eight (6.4%); sigmoid colon, 43 (34.4%); and rectum, 12 (9.6%). The nuclear grade was intermediate for 113 carcinomas, well differentiated for one, and anaplastic for 11. The mean (SD) number of lymph nodes evaluated by H&E for each patient was 14 (8.4). Thirty three per cent (39 of 118) of the patients' primary carcinomas had histological evidence of blood vessel involvement with tumour cells, whereas perineural invasion was seen in just 7% (eight of 117) of subjects. Analysis of the primary carcinomas revealed that Ki-ras mutation was present in 33% (41 of 125); APC LOH was present in 45% of informative carcinomas (49 of 108); and DCC LOH was present in 69% of informative lesions (67 of 97). Fifteen per cent (19 of 125) of the primary carcinomas had MSI, and of these cases, nine were located in the caecum, six in the ascending colon, two in the descending colon, and one each in the sigmoid and transverse colon.

Micrometastases as detected by immunohistochemical staining were present in 41% (43 of 106) of cases (fig 1). Patients with micrometastases had a mean (SD) of 2.2 (1.3) positive nodes detected. There were significantly more lymph nodes removed and stained with H&E among the patients positive for micrometastases than in those negative for micrometastases (mean, 15 v 11; Mann-Whitney U; p = 0.002). The number of carcinomas with complete

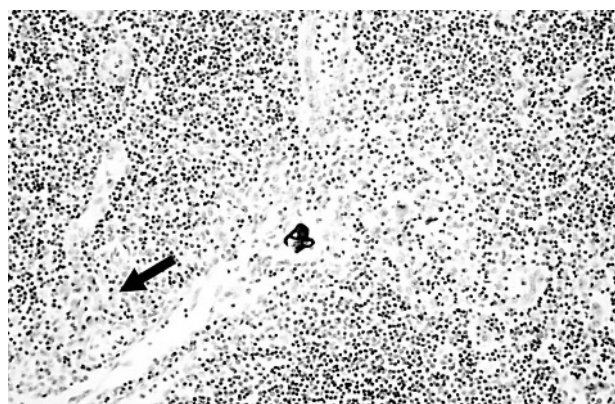


Figure 1 Light micrograph of a pericolic lymph node. The arrow indicates a cluster of metastatic colon carcinoma cells stained with monoclonal antibodies to keratin.

Table 1 Cross tabulation between molecular genetic changes and blood vessel and perineural involvement and micrometastases, controlling for the number of lymph nodes removed

Molecular genetic changes	Blood vessel involvement		Perineural involvement		Micrometastasis	
	N*	%†	N*	%†	N*	%†
APC						
LOH	15/44	34%	4/44	9%	16/40	40%
No LOH	17/57	30%	2/56	4%	22/53	42%
RR	1.21		2.70		0.90	
p Value	0.65		0.25		0.82	
CI	0.52 to 2.82		0.47 to 15.48		0.38 to 2.16	
Ki-ras						
Mutated	11/40	28%	1/38	3%	9/34	26%
Wild-type	28/78	36%	7/79	9%	34/72	47%
RR	0.69		0.28		0.44	
p Value	0.39		0.23		0.08	
CI	0.30 to 1.60		0.03 to 2.42		0.18 to 1.11	
DCC						
LOH	17/63	27%	4/64	6%	24/57	42%
No LOH	14/27	52%	2/26	8%	7/25	28%
RR	0.34		0.80		1.72	
p Value	0.02		0.81		0.33	
CI	0.13 to 0.89		0.14 to 4.66		0.58 to 5.07	
Microsatellite						
Unstable					7/16	44%
Stable					36/90	40%
RR					0.89	
p Value					0.85	
CI					0.29 to 2.79	

*The number of carcinomas listed with each histological change is less than the total number studied because some lesions had incomplete data or were uninformative; †reflects the percentage of carcinomas with information about the listed histological change and also with or without the particular molecular change.
APC, adenomatous polyposis coli; CI, confidence interval; DCC, deleted in colon cancer; LOH, loss of heterozygosity; RR, relative risk.

information for micrometastasis and for blood vessel and perineural tumour invasion was less than the total number of primary lesions. Furthermore, some carcinomas were uninformative with the markers used for APC and DCC LOH. Therefore, the cross tabulations of the histological findings with the molecular genetic changes have variable numbers in each group (table 1). Lymph node micrometastases were not associated with blood vessel involvement (p = 0.7) or perineural invasion (p = 0.9) (data not shown). Lymph node micrometastases were not associated with APC LOH, DCC LOH, Ki-ras mutation, or microsatellite instability, even when controlling for the number of lymph nodes removed (table 1). There was no difference between the type of Ki-ras mutation and the presence or absence of micrometastasis (data not shown). Blood vessel and perineural involvement by tumour cells was not associated with Ki-ras gene mutations or with LOH of the APC gene. However, blood vessel involvement was less frequent in those patients whose primary carcinomas revealed LOH of the DCC gene (table 1).

We also analysed whether the histological and immunohistochemical features or molecular changes of the primary carcinoma were associated with the size of the tumour. Using the gross measurements in three dimensions, the volume of

the tumour was calculated and grouped into tertiles, namely: < 11.5 cm³, between 11.5 and 32 cm³, and > 32 cm³. Cross tabulations between this categorical variable for size and the following variables revealed no significant associations: blood vessel involvement with tumour (p = 0.8), perineural invasion with tumour (p = 0.7), Ki-ras mutation (p = 0.3), APC LOH (p = 0.09), DCC LOH (p = 0.2), and micrometastases (p = 0.2)

Survival analysis was based on a mean (SD) follow up interval of 7.1 (3.9) years. Survival information was not available for two patients. There were 21 patients who died with evidence of disease. None of the histological or molecular variables considered had a significant impact on either overall survival (data not shown) or on death with disease (table 2). In particular, for the 89 patients with complete molecular data for APC, DCC, and Ki-ras, there was no significant effect on overall survival whether the primary carcinoma had an abnormality in one (p = 0.7), two (p = 0.8), or three (p = 0.9) of these genes. The addition of micrometastases to the effect of the three molecular markers plus blood vessel involvement did not reveal a significant effect on survival, even controlling for the number of lymph nodes removed (p ≤ 0.36).

Table 2 Survival analysis comparing those who died with evidence of metastatic colorectal carcinoma (21 patients) with all others

Variables	Number	Hazard ratio	p Value
Age	120	1.01	0.52
Sex (female/male)	61/59	1.31	0.55
Blood vessels involved (no/yes)	77/36	0.45	0.21
APC (normal/LOH)	57/47	1.88	0.20
DCC (normal/LOH)	29/64	0.91	0.86
Ki-ras (normal/mutation)	81/39	0.84	0.73
Micrometastasis	61/42	1.02	0.97

APC, adenomatous polyposis coli; DCC, deleted in colon cancer; LOH, loss of heterozygosity.

Table 3 Reports of micrometastases and survival in patients with Dukes's B colorectal cancer

Ref	No patients	% Patients with MM	Lymph nodes/pt*	Positive nodes/pt	Follow up (months)	IHC markers	Result
13	100	39%	4.7	2.0	57	CK8/18/19	NS
17	93	31%	19.4	1.8	66	CK MNF 116	NS
18	77	25%	7.2	NA	120	CK AE1/AE3	NS
19	46	26%	13	2.0	60	CEA, CK AE1/AE3	NS
14	50	28%	11.3	2.3	66	CK AE1/AE3; TAG-72	S
15	100	34%	11.5	NA	42	CK20 (PCR)	S
16	42	21.4%	15.3	2.1	120	CK8/18	S
Our study	106	41%	14 (8.4)	2.2	85	CK AE1/AE3; CK8/18	NS

*Mean (SD).

CEA, carcinoembryonic antigen; CK, cytokeratin; EMA, epithelial membrane antigen; IHC, immunohistochemistry; MM, micrometastases; NS, not significant; pt, patient; S, significant; TAG-72, tumour associated glycoprotein-72.

DISCUSSION

A micrometastasis is defined as a single tumour cell or a very small cluster of tumour cells within a lymph node, measuring less than 2 mm in diameter.¹³ These cells may be identified immunohistochemically, but are too few in number to be detected by routine H&E staining. Published reports evaluating lymph nodes for micrometastases have used monoclonal antibodies against different markers of epithelial histology, such as carcinoembryonic antigen, epithelial membrane antigen, cytokeratin AE1/AE3, keratin polypeptide CAM 5.2, or tumour associated glycoprotein-72. Several studies have shown micrometastases to have an adverse effect on survival in stage B colon carcinoma,¹⁴⁻¹⁶ but others have failed to find such an association (table 3).¹³⁻¹⁷⁻¹⁹ This discrepancy is difficult to explain, because the studies were similar in sample size, percentage of patients with micrometastases, number of positive lymph nodes for each patient, and duration of follow up. However, they do differ with regard to which immunohistochemical markers were used and in the number of different markers used, and to some extent in the number of lymph nodes evaluated for each patient. Two of the studies showing significance for micrometastases had a limited follow up interval,¹⁴⁻¹⁵ and the third had a small study group.¹⁶ The results of our study are consistent with those studies showing no significant association between micrometastases in lymph nodes and survival.

In 1990, Fielding *et al* recommended the examination of at least 12 lymph nodes for each surgical colon resection.²⁰ More recent studies have shown that by increasing the number of lymph nodes examined, the number of patients identified with lymph node metastases on routine H&E staining increases. Therefore, these patients are staged and treated differently, and overall disease free survival is improved for those remaining patients with lymph nodes negative by

H&E.²¹⁻²² A recent secondary analysis of an intergroup trial of adjuvant chemotherapy for colon cancer also showed an increase in survival as greater numbers of lymph nodes were analysed.²³ Therefore, rather than looking specifically for micrometastases, it may be simpler and more informative to maximise the number of lymph nodes removed during surgery for routine H&E staining. Of interest, the patients in our study for whom micrometastases were detected had significantly more lymph nodes removed and stained.

“The results of our study are consistent with those studies showing no significant association between micrometastases in lymph nodes and survival”

There are no studies in the literature correlating molecular changes of the primary tumour with the presence or absence of micrometastases. We found that Ki-ras mutation and APC or DCC LOH in the primary carcinoma had no effect on the incidence of micrometastases.

The evidence suggests that there are currently insufficient data to justify using molecular genetic changes in primary colorectal carcinomas as prognostic markers. Furthermore, micrometastases do not provide consistent prognostic information on survival. However, our data and the results of others clearly show the value of increasing the numbers of lymph nodes removed and analysed along with the primary tumour. We recommend that a minimum of 12 lymph nodes should be evaluated for metastases by H&E in all cases of primary colorectal carcinoma resection. It may be that in the immediate future, older methods of pathological analysis will continue to be the most informative.

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Take home messages

- Molecular genetic changes, such as Ki-ras gene mutations, loss of heterozygosity of the APC and DCC genes, and microsatellite instability, do not appear to be useful prognostic markers in primary colorectal carcinoma
- Micrometastases do not provide consistent prognostic information on survival
- However, increasing the numbers of lymph nodes removed and analysed along with the primary tumour increases the number of metastases identified, and a minimum of 12 lymph nodes should be evaluated by haematoxylin and eosin in all primary colorectal carcinomas resected

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ECHO

GPs prefer more direction from laboratory reports on vaginal discharge



Please visit the *Journal of Clinical Pathology* website [www.jclinpath.com] for a link to the full text of this article.

Narrowing the gap between the information doctors want from high vaginal swab (HVS) samples and what laboratories currently provide could improve the management of vaginal discharge in general practice, according to a survey in England.

Around three quarters of the 500 plus general practitioners (GPs) responding wanted laboratory reports to suggest a diagnosis based on microbiological results—which six PHLS laboratories in their area do—and to suggest treatment—which only two do and some consider is not their remit. Three quarters of the GPs said they would take an HVS sample for vaginal discharge in a young woman, and three quarters would request microscopy, culture, and antibiotic sensitivity testing.

Responses from the laboratories indicated wide variation in processing and reporting on such samples; a national guideline on processing could at least address some disparities, as there is no existing PHLS standard.

The survey asked 2146 GPs in North Thames region how they would investigate and manage a theoretical case—a 20 year old woman taking the combined oral contraceptive pill with two weeks' vaginal discharge after changing her male sexual partner. The 22 laboratories in the region were asked specific questions about how they processed and reported HVS samples.

In 2002 the government advocated greater GP input in managing sexually transmitted diseases, and processing HVS samples is a large component of PHLS work. GPs' expectations of the test and whether they are fulfilled are largely unknown, but it seems that more specific advice might be a cost effective improvement.

▲ Noble H, *et al.* *Sexually Transmitted Infections* 2004;**80**:204–206.



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