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Detection of exfoliated carcinoma cells in colonic luminal washings by identification of deranged patterns of expression of the CD44 gene

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Abstract

Aims—**To investigate whether colonic cancer cells exfoliated into the lumen of the organ can be detected by identification of their abnormal CD44 gene products.**

Methods—**Exfoliated cells were obtained by centrifugation of saline wash-outs of 27 surgically resected colon specimens obtained from 15 patients with carcinoma, seven with ulcerative colitis and five with Crohn's disease. After extracting cellular mRNA, amplification by the reverse transcription - polymerase chain reaction (RT-PCR) technique and analysis by Southern blot hybridisation was carried out to examine the levels and patterns of transcription of exons 11(v6), and 12(v7) and intron 9 of the CD44 gene. The transcription of these CD44 components was also examined by RT-PCR of snap-frozen solid tissue specimens from 11 of the above patients with colorectal carcinoma, seven with ulcerative colitis and five with Crohn's disease.**

Results—**Abnormal expression of exons 11(v6) and 12(v7) was detected in exfoliated cells from 11 (73%) of 15 patients with carcinoma, but not in any patients with inflammatory bowel disease (IBD). The retention of intron 9 in CD44 mRNA transcripts was detected in washings from four (27%) carcinoma specimens but not in washings from non-malignant specimens. It was confirmed that in solid tissue samples from the same carcinomas there was abnormal over-expression of numerous alternatively spliced CD44 species containing transcripts of exons 11 and 12 and retention of intron 9. Low level expression of these exons was detected in tissue from inflammatory lesions from five of seven patients with ulcerative colitis and four of five with Crohn's disease. The retention of intron 9 was not seen in normal mucosa nor IBD.**

Conclusion—**Abnormal expression of the variant exons and of intron 9 of the CD44 gene in tumour cells exfoliated into the colonic lumen may be helpful markers for the early, non-invasive, diagnosis of colorectal cancer.**

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Colorectal carcinoma is one of the most common fatal neoplasms in many countries. Surgical treatment is very effective if the tumour is removed before it has spread but, despite recent advances in the clinical investigation of bowel disorders, including double contrast barium enema and endoscopy, the early detection of colorectal carcinoma is still a major clinical problem. The above methods are too expensive, uncomfortable and labour intensive to be suitable for widespread regular use. The presence of fresh or occult blood in the stool is a frequent feature in symptomatic disease but is too non-specific to be useful for screening and/or detection of early pre-invasive disease. A more reliable, preferably non-invasive, method is therefore urgently needed for routine evaluation of symptomatic and asymptomatic individuals. The deranged expression of the CD44 gene, frequently observed in various types of tumours, including ones of the stomach,[1–3] colon,[4–6] breast,[7 8] and uterus,[9] is a promising candidate for this purpose.[6–8]

CD44 is a widely distributed, receptor type protein which exists as various isoforms generated by alternative splicing of the products of at least 10 variant exons. Many diverse functions have been ascribed to this family of proteins, including involvement in lymphocyte traffic[10 11] and adhesion of cells to their neighbours or the adjacent extracellular matrix. We have recently demonstrated the feasibility of non-invasive detection of bladder cancer by identification of abnormal CD44 mRNA and protein in exfoliated cancer cells in urine.[12–14] This work also identified a new exon of this gene, designated 9a, and reported that retention of intron 9, a non-coding section of the gene, is seen in cytoplasmic mRNA transcripts extracted from urine cell pellets of about 60% of patients with bladder cancer but in only one (2·5%) of 42 controls.[14] In a further study it was found that over-abundance of CD44 transcripts containing portions homologous to exons 11(v6) and 12(v7) and intron 9 occurred in 80% of colorectal carcinomas.[15] These results suggested that abnormal CD44 gene expression might provide a valuable new tool in clinical gastroenterology for the early detection and monitoring of malignancy and we decided to examine whether cancer cells exfoliated into the colonic lumen could be detected by these sensitive techniques. To investigate this, we have used saline washings of the lumen of

Table 1 Clinicopathological findings of patients with cancer and the results of molecular analysis

Case number	*Age (years)*	*Sex*	*Location*	*Histology**	*T*	*N*	*Dukes' stage*	*Cytology*	*FOB*	*CD44*
1	66	M	S	Mod	3	0	B	–	–	–
4	54	M	A	Muc	2	2	C	–	+	+
24	47	F	R	Well	3	1	C	s	+	+
26	73	M	R	Poor	4	2	C	–	ND	+
29	72	M	C	Mod	3	2	C	ND	+	+
32	65	F	R	Mod	3	1	C	–	+	+
36	56	M	R	Poor	4	2	C	–	ND	+
37	60	M	C	Muc	3	0	B	–	ND	–
38	77	F	A	Mod	3	0	B	–	–	–
39	82	M	A	Mod	3	1	C	s	+	+
40	83	M	S	Mod	3	0	B	ND	–	–
50	59	F	S	Mod	3	1	C	+	+	+
51	59	F	R	Mod	3	1	C	–	+	+
52	68	M	R	Mod	4	0	B	s	ND	+
53	67	F	R	Mod	3	0	B	+	+	+

S = sigmoid colon; A = ascending colon; C = caecum; R = rectum.* Histology: T and N were followed according to UICC classification. Well = well differentiated adenocarcinoma; Mod = moderately differentiated adenocarcinoma; Poor = poorly differentiated adenocarcinoma; Muc = mucinous carcinoma; ND = not done; s = suspected; FOB = faecal occult blood.

specimens resected from patients with colorectal cancer and inflammatory bowel disease (IBD) to examine the expression of CD44 exons 11 and 12 and intron 9 by the reverse transcription–polymerase chain reaction (RT-PCR) technique. The analysis of the expression of these genes in non-malignant ulcerative lesions of the colon was undertaken to investigate the specificity of the changes observed.

Methods

PROCUREMENT OF EXFOLIATED CELLS BY COLONIC LUMINAL WASHING

Fifteen colorectal cancer, seven ulcerative colitis and five Crohn's disease specimens were used to collect exfoliated cells. The lumen of each surgically resected specimen was washed with tap water to clear faecal debris and then with 500 ml physiological saline before opening for pathological examination and sampling. A 1 ml aliquot of the saline washings was removed for cytology after which the remainder was centrifuged at 3000 rpm for 15 minutes. The resulting cell pellets were collected, snap-frozen in liquid nitrogen and stored at −80°C. Fresh tissue specimens from carcinomas, corresponding normal mucosa and from IBD lesions were snap-frozen and stored in liquid nitrogen until use. The presence of carcinoma or of inflammatory lesions in tissues taken from resected specimens was routinely confirmed by cryostat sectioning before molecular analysis.

RT-PCR AND SOUTHERN HYBRIDISATION

Total cellular RNA was extracted from tissue samples by the acid guanidium phenol-chloroform method and mRNA was obtained from colonic washing samples using Microfast Track extraction kits (Invitrogen, Leek, The Netherlands). cDNA was synthesised with reverse transcriptase followed by amplification by PCR with cDNA Cycle Kits (Invitrogen), as described previously.[12] Total RNA (5 μg) from tissue specimens or 50 ng poly A^+ selected RNA from colonic washings was used for the RT-PCR reaction. The conditions of PCR were as follows: 94°C for five minutes and 85°C for one minute, during which time Taq polymerase was added (hot start) and 30 cycles of 94°C for one minute, 55°C for one minute and 72°C for two minutes followed. The primers used were as follows: P1: 5′-GACACATATTGCTTCAATGCTTCAGC-3′; P2: 5′-CCTGAAGAAGATTGTACATCAGTCACAGAC-3′; I4: 5′-GTAATGGGTTCTGCATATTTAATGAA-3′; AI4: 5′-CTGTGATGATGGTTAAATACACTG-3′; EX10: 5′-TCCAGGCAACTCCTA-3′; AEX10: 5′-CAGCTGTCCCTGTTG-3′; E3: 5′-AGCCCAGAGGACAGTTCCTGG-3′; E5: 5′-TCCTGCTTGATGACCTCGTCCCAT-3′; A15: 5′-ACTGGGGTGGAATGTGTCTTGGTC-3′; and P4: 5′-GATGCCAAGATGATCAGCCATTCTGGAA-3′.

Ten microlitres of the 50 μl PCR reaction mixture was electrophoresed in a 1.2% agarose gel and transferred to a Hybond N^+ (Amersham, Little Chalfont, UK) nylon membrane with 0·4 N NaOH solution overnight and hybridised with probes made by PCR (35 cycles, 94°C for one minute, 55°C for one minute, 72°C for two minutes) using the primers listed above from CD44 genomic clone C2311.[14] These probes were labelled with peroxidase, using ECL direct nucleic acid labelling to produce chemiluminescent probes and detected with the ECL detection system (Amersham). The positions to which the primers anneal are shown in fig 1. The probes sizes used for each exon were as follows: exon 11, 129 base pairs (bp); exon 12, 75 bp; intron 9 probe, 475 bp; and standard probe (P2-A15), 321 bp. The conditions used for hybridisation, washing and detection were those recommended by the manufacturer's protocol.

Results

TISSUE SAMPLES

We initially examined the patterns and levels of expression of sequences homologous to CD44 exons 11 and 12 and intron 9 in samples of carcinoma tissue and corresponding normal mucosa from 11 of the patients whose luminal washings we had collected. The transcription of these sequences was also examined in inflammatory lesions from seven patients with ulcerative colitis and five with Crohn's disease. These were essential preliminary examinations to provide a firm basis for the work on the colonic washings. It was confirmed that in these carcinoma samples there was over-expression

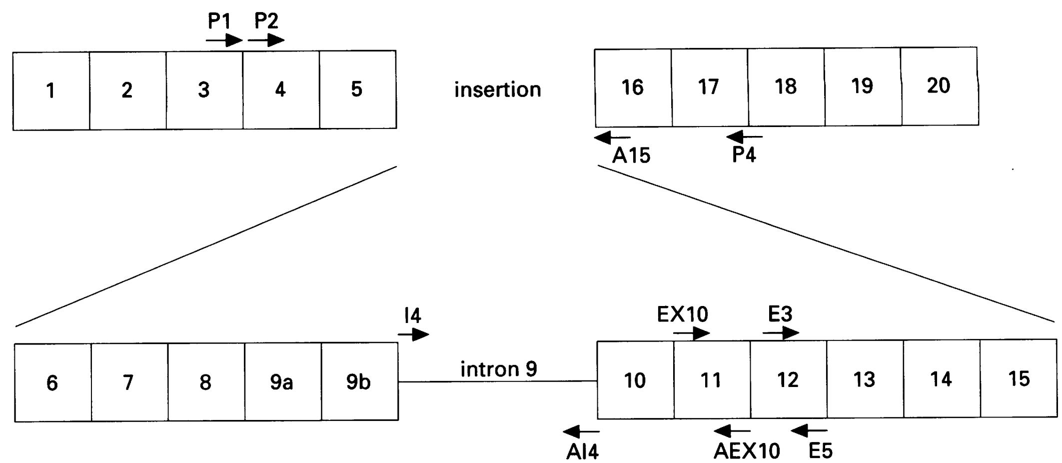

Figure 1 Structure of the CD44 gene products showing the positions to which the primers anneal. The probe for the standard portion of the gene was synthesised by PCR using P2 and A15 on a DNA template consisting of the PBS IS clone in Bluescript plasmid. This construct contains a PCR product (482 bp) obtained from human peripheral blood lymphocyte cDNA using primers P1 and P4. Probes for exons 11 and 12 and intron 9 were generated by PCR using specific primers to amplify portions of a template consisting of a human CD44 genomic clone (C2311).

of numerous alternatively spliced CD44 mRNA species containing transcripts from several of the exons in the variable region of the gene, as shown in fig 2. This shows that the expression of CD44 exons 11 and 12 was noticeably deranged in the tumour tissues of cases 51, 52 and 53, while it was undetectable or very weak in corresponding normal mucosa. The high molecular weight smear pattern of expression of these exons, which we have previously described to be characteristic of tumour tissue, was observed in all carcinoma samples examined in the present study. The transcription of exons 11 and 12 was also detected in inflammatory lesions of five specimens of ulcerative colitis and four of Crohn's disease, but the pattern of expression of the variant exons in carcinoma tissues was clearly different from that in the inflammatory lesions.

Analysis of the mRNA obtained from each specimen, for the possible retention of intron sequences in the CD44 transcripts, showed that although the level of representation of such non-coding sequences in this material was weak compared with that of exons 11 and 12, the presence of intron 9 was detected in mRNA from all carcinoma tissues but it was not detected in normal tissue nor IBD lesions (fig 2).

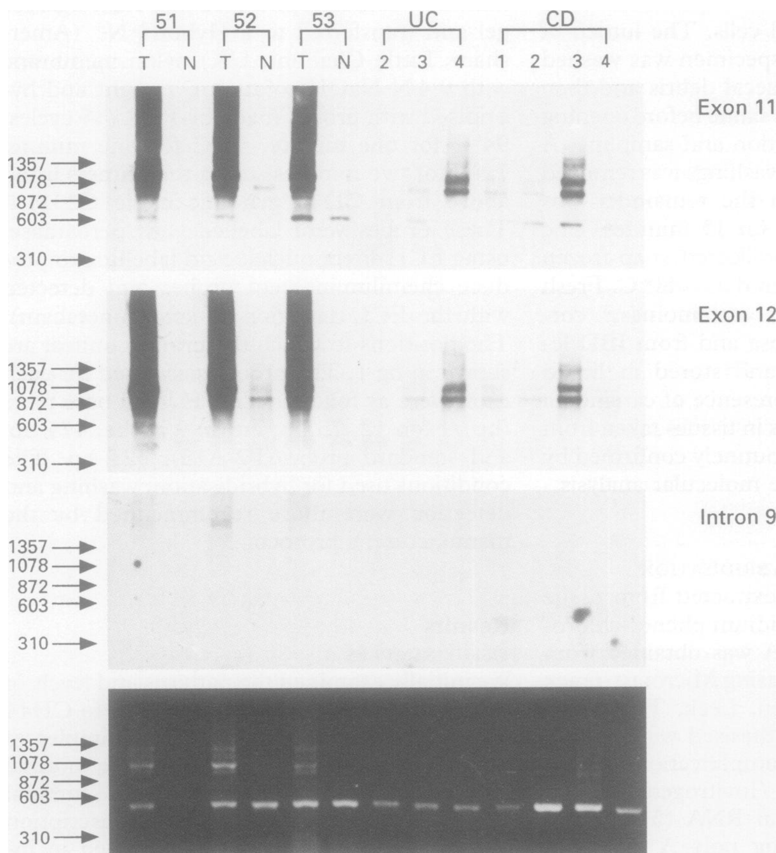

Figure 2 Southern blot hybridisation analysis of RT-PCR amplification products obtained using P1 and P4 primers on cDNA from colon carcinoma tissues (T) and corresponding normal mucosa (N) from cases 51, 52, 53, and inflammatory lesions of patients with ulcerative colitis (UC) and Crohn's disease (CD). The same filter was sequentially hybridised with probes for exons 11 and 12 and intron 9 to show the transcriptional status of these parts of the gene. The ethidium bromide staining of the gel is presented in the bottom panel.

EXFOLIATED CELLS FROM COLONIC WASHINGS

The results of RT-PCR/Southern hybridisation using RNA from pelleted exfoliated cells of the cases shown in fig 2 are presented in fig 3. The exfoliated cells in the washings from cases 51, 52 and 53 strongly expressed a smear pattern, with an accentuated single band, containing transcripts of exons 11 and 12. In case 50 a similar pattern of expression of these exons is also clearly observed, although it is weak. The intensity of the abnormal expression pattern might depend on the number of cancer cells in the washings.

This unusual pattern of expression, similar to that seen in tumour tissue samples (fig 2), was observed in the exfoliated cells washed from 11 (73%) of the 15 patients with cancer. Comparable changes were not seen in any of the washings from non-neoplastic colon specimens, but the single 1100 bp accentuated band was detectable in the cells pelleted from the washings of one patient with Crohn's disease (track CD1, fig 3).

The retention of intron 9 in CD44 mRNA was detected in cell pellets from four (27%) of 15 patients with cancer, but not in any of the

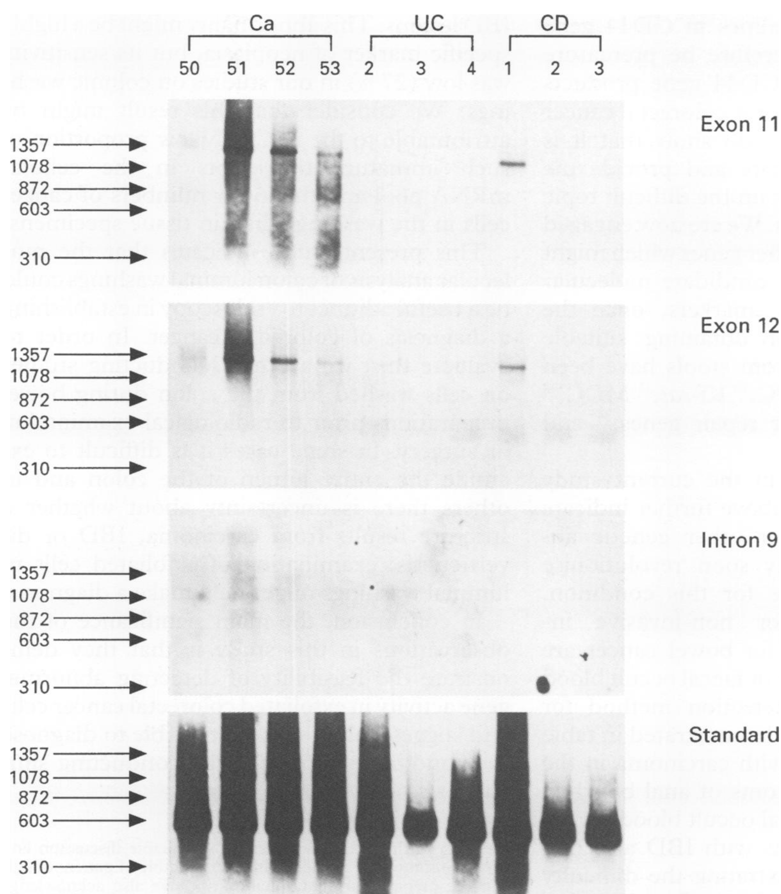

Figure 3 The results of Southern blot hybridisation analysis of RT-PCR amplification products using exfoliated cells from colon luminal washings. The results of hybridising the same filter sequentially with probes for exons 11 and 12, intron 9, and standard form CD44 are presented. Ca=patients with carcinoma; UC=patients with ulcerative colitis; CD=patients with Crohn's disease. The numbers above the lanes are case numbers.

specimens from patients with IBD. This is illustrated in fig 3, which shows the results of sequentially hybridising the same filter with probes for various sequences. Although the intensity of the signal observed after hybridisation of the filter with the intron 9 probe indicates that the proportion of transcripts containing this sequence was low, the results show that it was unequivocally present in the RNA from exfoliated cells obtained from cases 51 and 52. The intensity of the signal obtained with intron 9 in each patient correlated with that obtained with the probes for exons 11 and 12.

Pertinent clinicopathological information relating to these patients, including their faecal occult blood status and the results of cytopathological evaluation of the exfoliated cells in their colonic washings are listed in table 1. Most of the patients presented positive with faecal occult blood tests regardless of whether they had IBD or colonic neoplasia. Also, the cytopathological appearances of the exfoliated cells were usually difficult to interpret because of poor preservation. There was no relation between TNM stage, degree of differentiation or other clinicopathological features of these tumours and the findings on molecular analysis of the luminal washings. The histological sections of patients with IBD, examined for routine diagnostic purposes, showed no dysplastic lesions (data not shown).

Discussion

It is of great interest and practical importance to determine whether exfoliated cancer cells can be detected in clinical specimens obtained non-invasively, or minimally invasively. We have previously demonstrated that abnormal quantities of unusual CD44 mRNAs and proteins were detected in exfoliated cells in naturally micturated urine from patients with bladder cancer.[12 13] The frequency and consistent occurrence of these abnormalities enabled the laboratory to identify patients with bladder cancer with 91% sensitivity and 83% specificity using RT-PCR. Western blot analysis, with an antibody directed against CD44, on a separate set of patients resulted in identification of patients with carcinoma with 75% sensitivity and 100% specificity. The group of patients with cancer in each of these separate studies included those with early pre-invasive carcinomas and well differentiated papillary tumours, indicating that non-invasive, early detection of cancer using these molecular techniques is feasible.

In the present study, the abnormal expression pattern of CD44 exons 11 and 12, typical of neoplasia, was detected in 73% of the luminal washings from patients with carcinoma, suggesting that the detection of exfoliated colorectal cancer cells, by the identification of irregularities in CD44 expression, can be achieved with reasonable accuracy. The clinical implication of this result is that the application of this technique to evacuated stool specimens could result in a new non-invasive test for colorectal cancer. In preliminary experiments we have successfully detected abnormal CD44 expression using RT-PCR on mRNA from exfoliated cells in stools from a small group of patients (six of 11) with colorectal cancer, but not in ones from normal subjects (Matsumura and Tarin, unpublished observations). However, for the sensitivity and specificity of this approach to be evaluated accurately, it will be necessary to develop reliable techniques for retrieving viable exfoliated cells from evacuated stools. Clinical bowel preparation regimens which render the stools liquid and evacuate the lumen in preparation for surgery or radiological and endoscopic examination may be helpful for this purpose.

The proportion of patients with colorectal cancer in whom we have identified disturbances in CD44 gene expression in the colonic luminal contents, and thus inferred the presence of exfoliated cancer cells, compares favourably with figures published in previous reports, using other molecular markers. For example, p53 gene mutations were detected in 1–7% of exfoliated cells in the urine of three patients with bladder cancer,[16] whilst H-*ras* gene mutations were observed in the urinary cells of 10 of 21[17] patients with the same condition. K-*ras* mutations were found in DNA retrieved from stool samples from eight of nine cases with colorectal cancer.[18] However, the encouraging figures in our present report could be attributable to the mode of obtaining exfoliated cells—that is, by washing out excised colons, or to the relative simplicity of the techniques

for detection of abnormalities in CD44 gene expression. It would therefore be premature to claim that abnormal CD44 gene products constitute the best analyte for colorectal cancer detection, but the results do show that it is a very promising candidate and provide the incentive for further work on the difficult topic of defecated stool analysis. We are now engaged in such investigations. Other genes which might deserve consideration as candidate molecular diagnostic or prognostic markers, once the technical procedures for obtaining suitable yields of suitable cells from stools have been solved, include p53,[19] APC,[20] Ki-*ras*,[21] MCC,[22] DCC,[23] replication error repair genes,[24] and telomerase.[25 26]

The results obtained in the current study and in others described above further indicate that tests for this and/or other genetic abnormalities will probably soon revolutionise the diagnostic repertoire for this condition. Traditional methods for non-invasive investigation or screening for bowel cancer are extremely crude. Testing for faecal occult blood is unsatisfactory as a detection method for colorectal carcinomas as demonstrated in table 1. Most of the patients with carcinoma in the present study had symptoms of anal bleeding or tested positive for faecal occult blood. However, most of the patients with IBD also had the same symptoms, illustrating the difficulty of discriminating between patients with cancer and those with IBD. Cytology is a powerful and reliable method for identifying cancer cells, but it is labour intensive, expensive and depends heavily on the quality of preservation of the cells in the sample. It has not been found to be useful for the diagnosis of colorectal cancer, as demonstrated above. The data now accumulating indicate that molecular genetic analytical methods are poised to offer more specific and informative diagnostic alternatives.

Although this work has shown that the level and pattern of expression of exons 11 and 12 of the CD44 gene were deranged in tumour tissues compared with IBD lesions, it is important to note that orderly, low level expression of these exons similar to that seen in extracts of normal tissues was detectable in the samples from the non-neoplastic diseases (fig 2). Rosenberg *et al*[27] have reported recently that raised expression of CD44 variant exon 3 (exon 8) and variant exon 6 (exon 11) encoded epitopes was detected immunohistochemically on colonic crypt epithelial cells in 23 of 25 ulcerative colitis samples compared with three of 18 samples of colonic Crohn's disease. The data presented here show that using RT-PCR, it is easy to distinguish between the normal, inflammatory and neoplastic patterns of expression of the variable region of this gene, both in tissues and in exfoliated cells.

The over-abundant transcripts produced by the CD44 gene in tumour cells contain immature or defective mRNA species of a wide range of sizes, with retained intronic sequences.[14 15] This additional distinguishing feature was confirmed in all the tumour tissues examined in this study, which has verified that intron retaining transcripts are not detected in IBD lesions. This abnormality might be a highly specific marker of neoplasia, but its sensitivity was low (27%) in our studies on colonic washings. We consider that this result might be attributable to the relatively low proportion of such immature transcripts in the cellular mRNA pool and the lower numbers of cancer cells in the washings than in tissue specimens.

This present study indicates that the molecular analysis of colon luminal washings could be a useful adjunct to endoscopy in establishing a diagnosis of colorectal cancer. In order to evaluate this, we are now conducting studies on cells washed from the colon during bowel preparations prior to radiological examination or surgery. In some cases it is difficult to examine the entire lumen of the colon and in others there is uncertainty about whether a stricture results from carcinoma, IBD or diverticulitis; examination of exfoliated cells in luminal washings might help make a diagnosis.

In conclusion, the main significance of the observations in this study is that they demonstrate the feasibility of detecting abnormal gene activity in exfoliated colorectal cancer cells and suggest that it may be possible to diagnose the condition successfully by conducting similar tests on evacuated stools.

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