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Abnormal Retention of Intron 9 in CD44 Gene Transcripts in Human Gastrointestinal Tumors[1]

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Abstract

We have recently identified a new exon of the CD44 gene and demonstrated abnormal retention of a noncoding section, intron 9, in mRNA from bladder carcinomas. To analyze this further, the present study examined CD44 gene expression in cell lines from 14 esophageal, 3 colonic, and 4 breast carcinomas and in fresh samples from 20 colorectal carcinomas and corresponding normal colonic mucosa, using reverse transcriptase followed by the polymerase chain reaction (RT-PCR). This confirmed that there was abnormal assembly of several exons of the gene in cell lines and in tumor tissues from these organs. However, the most striking new finding was that intron 9 was present in RNA from 11 esophageal, 3 colon, and 1 breast carcinoma cell line, respectively. This was confirmed by RNase and DNase digestion analysis. Moreover, it was detected both in nuclear and cytoplasmic mRNA fractions, indicating that abnormal splicing of pre-mRNA occurs in cancer cells. The abnormal retention of intron 9 in CD44 gene transcripts was also demonstrated in tumor tissues from 16 (80%) of 20 patients with colon carcinoma, but there was no correlation with Dukes' stage. The biological significance of these observations is not yet understood. However, it is clear that, as with the abnormal expression pattern of CD44 variant exons, intron 9 retention is a good-candidate molecular diagnostic tool for colorectal carcinomas.

Introduction

CD44[3] is a ubiquitous cell adhesion molecule, the overexpression of which is found in a variety of carcinomas, including those of the stomach (1–3), colon (4–7), breast (8, 9), uterus (10), bladder (11), and lung (12), as well as in hematopoietic malignancies (13). These observations indicate that it could have an important role in cancer diagnosis and prognostic evaluation. The gene consists of 20 exons, 10 of which can be alternatively spliced and assembled in conjunction with 10 constitutively expressed exons, to produce a variety of protein isoforms (14, 15). Posttranslational processing events, such as glycosylation, can result in further modifications, producing a diverse family of transmembrane proteins. The standard isoform (CD44s) encoded by the 10 constitutively expressed exons is present in almost all normal cells, while the distribution of other more complex variant isoforms (CD44v) occurs in a tissue-specific manner.

We have recently demonstrated that noninvasive detection of bladder cancer can be accomplished by identification of unusual CD44 mRNA (11) and protein (16) in exfoliated cancer cells in urine. With RT-PCR analysis, distinctive large molecular weight amplicons can be visualized from tumor cell-containing samples, although long, dense smears are the more usual indications of severely deranged gene transcripts. We reasoned that a possible explanation for this highly characteristic abnormality could be that the overabundant transcripts produced by the CD44 gene in tumor cells contain immature or defective mRNA species of a wide range of sizes, possibly with retained intronic sequences. During the investigation of this hypothesis, we recently cloned and identified a new 437-bp exon of CD44, which was named exon 9a. The former exon 9 was renamed 9b (17). Additionally, a unique new finding was that retention of intron 9, a noncoding section of the gene, was detected in about 60% of bladder cancer cells exfoliated in the urine (17). These findings stimulated us to examine the possibility that intron 9 retention in CD44 transcripts could be useful as a more general indicator of carcinoma diagnosis in other tissues. In the present study, we have examined whether abnormal retention of intron 9 is present in a number of human gastrointestinal cell lines and, more importantly, in human colon carcinoma tissues.

Materials and Methods

Cell Lines and Tissues. Fourteen human esophageal carcinoma cell lines were studied (TE-1,2,3,5,7,8,12, and 13, and KYSE- 180-209, 200-165, 520-95, 510-145, 450-157, and 520-91). The TE series of cell lines were provided by Dr. T. Nishihira (Tohoku University, Tohoku, Japan) and the KYSE series by Dr. M. Imamura (Kyoto University, Kyoto, Japan). Three human colon carcinoma cell lines, HT-29 (ATCC HTB 38), SW 480 (ATCC CCL 228), and C170 (donated by Professor R Baldwin, Nottingham University, Nottingham, United Kingdom), as well as four human breast carcinoma lines MDA-MB 435 (given by Dr. J. E. Price, M. D. Anderson Cancer Center, Houston, TX), MDA-MB 435-2C5, MDA-MB 435-4A4, and ZR 75-1 (ATCC CRL 1500), were also analyzed. RT112 human bladder carcinoma cells, which we have previously shown to exhibit abnormal CD44 expression including intron retention, was used as a control cell line. Cells were routinely cultured in RPMI 1640 (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% FCS at 37°C in an atmosphere containing 5% CO_2. Colorectal carcinoma tissue samples and corresponding normal colonic mucosa from surgical resection specimens were snap-frozen and stored in liquid nitrogen until use. The presence of carcinoma cells in tissues taken from colonic tumours was routinely confirmed by cryostat sectioning before analysis.

RT-PCR and Southern Hybridization. Total cellular RNA was extracted by the acid guanidinium-phenol-chloroform method, and mRNA was purified using Oligotex dT (Qiagen). cDNA was synthesized with reverse transcriptase, followed by amplification by PCR with cDNA Cycle Kit (Invitogen) as described previously (11). Five μg of total RNA or 100 ng of poly A-selected RNA was used for the RT-PCR reaction. The conditions of PCR were as follows: 94°C for 5 min and 85°C for 1 min during which time Taq polymerase was added (Hot start) followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The primers used were: P1, 5′-GACACATATTGCTTCAATGCTTCAGC-3′; P2, 5′-CCTGAAGAAGATTGTACATCAGTCACAGAC-3′; E1, 5′-TTGATGAGCACTAGTGCTACAGCA-3′; E2, 5′-CATTTGTGTTGTTGTGTGAAGATG-3′; D1, 5′-TACGTCTTCAAATACCATCTCAGC-3′; AD1,

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[3] The abbreviations used are: CD44s and CD44v, standard and variant isoforms of CD44, respectively; RT, reverse transcription.

5′-GGTGCTGGAGATAAAATCTTCATC-3′; SM1, 5′-CTGCCTCTTCACCCGGAGATC-3′; SM2, 5′-CTGTGATAACGATGATTGAAG-3′; EX8, 5′-TCAACCACACCACGGGCTTTTGAC-3′; AEX8, 5′-AGTCATCCTTGTGGTTGTCTGAAG-3′; I4, 5′-GTAATGGGTTCTGCATATTTAATGAA-3′; AI4, 5′-CTGTGATGATGGTTAAATACACTG-3′; EX9, 5′-GTAGACAGAAATGGCACCACTGCT-3′; AEX9, 5′-GCTTGTAGAATGTGGGGTCTCTTC-3′; EX10, 5′-TCCAGGCAACTCCTA-3′; AEX10, 5′-TCCAGGCAACTCCTA-3′; E3, 5′-AGCCCAGAGGACAGTTCCTGG-3′; E5, 5′-TCCTGCTTGATGACCTCGTCCCAT-3′; SD5, 5′-GATGTCACAGGTGGAAGAAGAGAC-3′; D5, 5′-TTCCTTCGTGTGTGGGTAATGAGA-3′; A15, 5′-ACTGGGGTGGAATGTGTCTTGGTC-3′; and P4, 5′-GATGCCAAGATGATCAGCCATTCTGGAA-3′. Ten μl of the 50-μl PCR reaction mixture were electrophoresed in a 1.2% agarose gel and transferred to Hybond N$^+$ (Amersham, Buckinghamshire, United Kingdom) nylon membrane with 0.4 N NaOH solution overnight and hybridized with probes made by PCR (35 cycles; 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min) using the primers listed above from CD44 genomic clone C2311 (17). These were labeled with peroxidase using ECL direct nucleic acid labeling 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 probe sizes used for each exon were: exon 7, 123 bp; exon 8, 124 bp; exon 9a, 437 bp; exon 9b, 111 bp; exon 10, 111 bp; exon 11, 129 bp; exon 12, 75 bp; exon 15, 101 bp; intron 9 probe, 475 bp; and standard probe (P2-A15), 321 bp. The conditions used for hybridization, washing, and detection were those recommended by the manufacturer's protocol.

Separation of Nuclear and Cytoplasmic mRNA. Poly (A)$^+$ selected RNA was extracted from nuclear and cytosolic fractions of various cell lines. The fractions were separated by the method described previously with some modifications (18). The cell pellets were suspended in 5 volumes of the pellet of mixture A [0.01 M Tris-HCl (pH 7.0), 0.15 M NaCl, 2mM $MgCl_2$, 0.5% NP40, and 0.2 unit/μl RNasin ribonuclease inhibitor (Promega)]. The cells were homogenized by 20 gentle strokes with a Dounce homogenizer on ice and spun down at 1000 × *g* at 4°C for 5 min. The supernatant was used to obtain the cytoplasmic RNA. The pellet was washed three times with washing buffer (60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 100 mM EGTA, 15 mM Tris-HCl, 300 mM sucrose, and 5% glycerol) and then used to extract nuclear RNA. For the RNase and DNase digestion analysis, 100 ng of poly (A)$^+$ selected RNA samples were digested with either 1 μg of RNase (Boehringer) or 0.1 unit of RQ1 RNase-free DNase (Promega) at 37°C for 20 min. After incubation, RNase/DNase was inactivated by incubation at 70°C for 10 min. The reaction mixtures were extracted with phenol-chloroform and precipitated with ethanol, followed by RT-PCR reaction.

Results and Discussion

The initial analysis was performed on several cell lines in order to facilitate experimental analysis of the origin, identity, and cellular distribution of the abnormal CD44 transcripts. This was followed by work on fresh tissue samples from surgical resection specimens to evaluate the clinical utility of the findings.

Analysis of Cell Lines. We examined whether retention of the CD44 intron 9 sequence occurs in the mRNA from 14 esophageal, 3 colon, and 4 breast carcinoma cell lines. PCR amplification products resulting from the use of primers P1 and P4 were analyzed for the expression of CD44 variant exons and intron 9. Using these primers, the amplification product obtained would be 482 bp if a sample expresses CD44s, and there would be several other products of different sizes if the sample was producing alternatively spliced transcripts (CD44v). Carcinoma cell lines expressed a range of variant exons. All esophageal carcinomas and colon carcinomas strongly expressed exons 11 and 12 of the CD44 gene, and an intense high molecular smear was also obtained with ZR 75-1 human breast cancer cells (Fig. 2*a*). The expression of several other exons was also remarkably high. However, the expression of all CD44v exons was very low in MDA-MB 435 breast carcinoma cells and their clonal derivatives MDA-MB 435-4A4 and 2C5 (data not shown), indicating that this abnormality is not invariably seen in all malignant cell lines.

The most striking observation was that hybridization signals were also detected with the intron 9 probe (Fig. 2*a*). Strong positive signals were seen in 11 esophageal carcinoma cell lines (TE-1, -2, -5, -7, -8, -12, and -13; KYSE-180-209, 200-165, 510-145, and 520-91), 3 human colon cell lines (HT-29, SW480, and C170), and in the ZR-75-1 human breast carcinoma cell line. Several single and merging bands of molecular weights above 1.3 kb were seen in PCR products from the RT112 cell line, but these were 1.5 kb or larger in the other cell lines. The size ranges of the signals observed with each cell line depend on the combinations of the alternatively spliced exons or retained introns in the mRNA. We have preliminary evidence that other introns can also be included in tumor mRNA transcripts (data not shown).

To exclude the possibility that the PCR products might result from amplification of contaminating genomic DNA, RNase and DNase digestions were performed on the extracts from HT-29 human colon carcinoma and TE-13 human esophageal carcinoma cells. As before, RT-PCR was performed using primers P1 and P4. As shown in Fig. 2*b*, intron 9 expression was not detected in the RNase digested mRNA (Fig. 2*b*, *Lane 2*), but it was present in DNase-digested mRNA (Fig. 2*b*, *Lane 3*) confirming the abnormal retention of intron 9 in the RNA fraction of the cells.

To elucidate whether intron 9 is retained in the cytoplasmic mRNA, we separately extracted the poly(A)$^+$ RNA from the nucleus and from the cytosol of HT-29 colonic carcinoma cells. Each fraction was subjected to RT-PCR as we have described above. Similar Southern hybridization patterns were obtained from the nuclear and cytoplasmic fractions when their PCR products were hybridized with the intron 9 probe (Fig. 2*b*, *Lanes 4* to *7*). This demonstrates that the defective transcripts in tumor cells do traverse the nuclear membrane to the cytosol fraction. If these transcripts were translatable, the

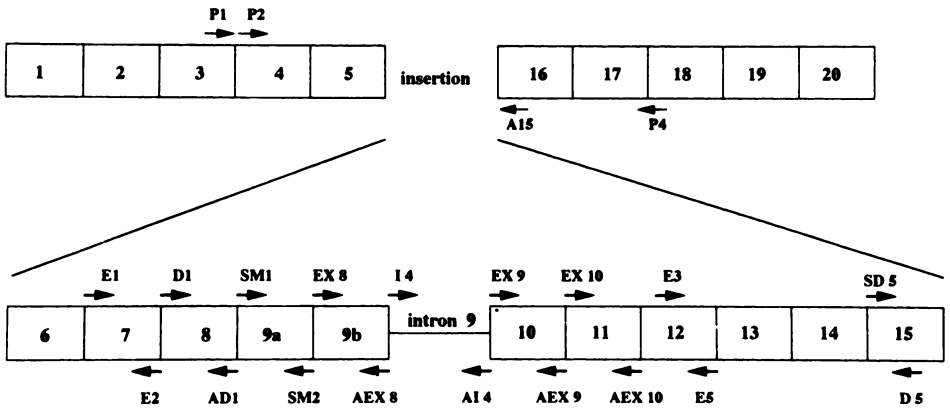

Fig. 1. Structure of the CD44 gene showing the positions to which the primers used anneal. The probe for transcripts from the standard portion of the gene was synthesized by PCR using primers P2 and A15 on a DNA template consisting of the PBS IS clone in Bluescript plasmid. This construct contains a PCR product (482 bp) obtained from human peripheral blood lymphocyte cDNA using primers P1 and P4. The resulting amplicon contains none of the variant exons. Probes for individual variant exons and for intron 9 were generated by PCR using specific primers to amplify portions of a template consisting of a human CD44 genomic clone (C2311).

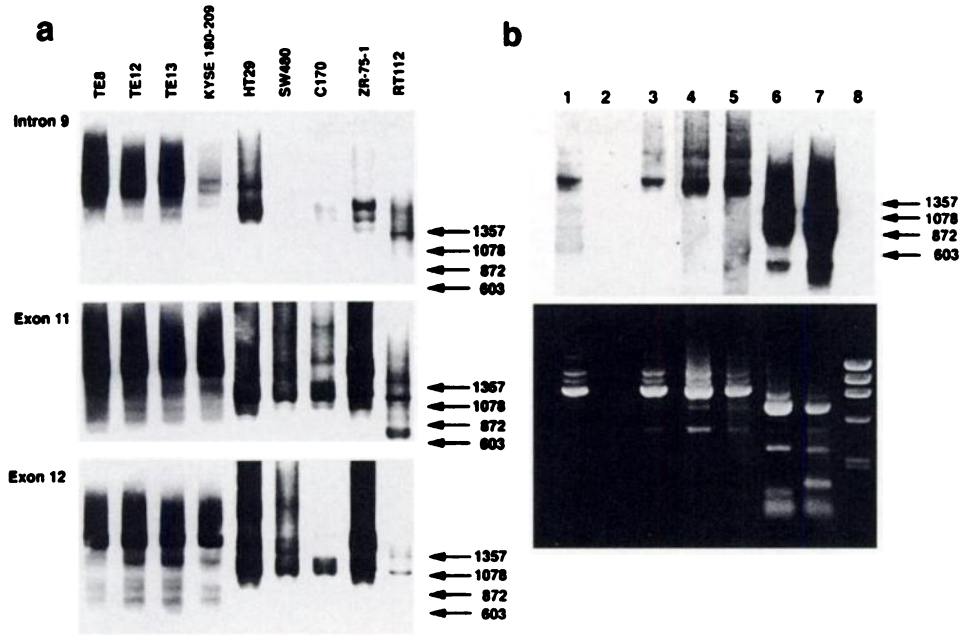

Fig. 2. *a*, Southern blot analysis of RT-PCR amplification products obtained using primers P1 and P4 on cDNA from human esophageal (TE-8,-12 and -13; KYSE-180-209), colon (HT-29, SW-480, and C-170), ZR-75-1 human breast and RT 112 human bladder carcinoma cell lines. The filter was hybridized with probes for exon 11, exon 12, and intron 9 and was stripped between each probing. *b*, results of RNase and DNase digestion analysis of HT29 human colon carcinoma cells are presented in *Lanes 1, 2*, and *3*. *Lane 1*, RT-PCR on untreated extracts; *Lane 2*, RT-PCR following RNase digestion of extracts; *Lane 3*, RT-PCR following DNase digestion of extracts. Poly(A)$^+$-selected RNA was digested with either RNase or DNase, followed by the RT-PCR reaction using primers P1 and P4. Southern blot analysis of the PCR products was performed using a probe for intron 9 for hybridization. Further analysis of the retention of intron 9 in mRNA transcripts in nuclear and cytoplasmic RNA fractions is presented in *Lanes 4–7*. These experiments used 100 ng poly(A)$^+$-selected RNA from HT29 human colon carcinoma cells. *Lanes 4* and *5*, P1, P4 primer set was used for the RT-PCR reaction. *Lanes 6* and *7*, E1, E5 primer set was used for the RT-PCR reaction. Cytoplasmic RNA was used in *Lanes 4* and *6*, and nuclear RNA was used in *Lanes 5* and *7*, respectively. Ethidium bromide staining of the gel is presented below the Southern blot hybridization autoradiogram. The marker used was Phi X 174 *Hae*III. The results show that the signal is abolished by RNase digestion of the extracts but not by DNase digestion, verifying that it is generated by amplification of mRNA transcripts containing the intron and that contamination with genomic DNA is excluded.

presence of intronic sequences could result in the incorporation of specific new peptide sequences into corresponding proteins, or in the formation of truncated variants, or in shifts in the reading frame downstream from the insert. Such shifts would alter the amino acid composition of the structure being assembled, giving rise to a family of uniquely unusual proteins.

Analysis of Colorectal Carcinoma Tissues. We have discussed previously the significance of abnormal CD44 gene expression (exon 12) for the diagnosis and clinical evaluation of colorectal carcinomas (4). In the present analysis of CD44 expression in human colon carcinoma and in normal colonic mucosa, we examined the identities and the patterns of expression of the variant exons most likely to be present in the alternatively spliced CD44 mRNA. We also assessed the frequency and level of retention of intron 9 sequences in these transcripts.

Twenty-four colon carcinoma specimens and their corresponding normal tissues were analyzed for the expression of CD44 variant exons using probes for exons 7, 8, 9a, 9b, 10, 11, 12, and 15. Five μg of total RNA was used for the RT-PCR reaction, and the products were electrophoresed and blotted. The same filters were stripped and rehybridized with each probe to ensure comparability. It was confirmed that in carcinoma samples, there was abnormal overexpression of numerous alternatively spliced CD44 mRNA species containing transcripts from several of the exons in the variable region of the gene. As demonstrated in Fig. 3, exons 11, 12, and 15 were more frequently expressed than the other exons in the tumor sample. Positive signals were obtained in 91% of tumors for exon 11, 82% for exon 12, and 88% for exon 15, respectively. The great majority of the tissues showed numerous ladder-like bands frequently fused to produce smears demonstrating overexpression of many specific CD44 mRNA species, as we have described previously (4). Probes for exons 11 and 12 discriminated clearly between tumor tissues and normal ones because in normals, the signals were very weak or absent. Furthermore, exon 15 expression was detected both in normal and tumor tissues but at much lower intensity in normals. Overexpression of exon 8 was seen in tumor tissues (79% of the samples), although it was also detectable in normal tissues. Positive signals were also detected in 54% of tumors with exon 7 and 10 probes, and in 21% with exon 9b. Expression of exon 9a was detected in only one specimen of tumor tissue. The degree of expression of CD44s in these samples is also shown in Fig. 3 for comparison with that of the other exons. From the above information, it can be deduced that probes for exons 11 and 12 are both good markers for tumor diagnosis, but further work is in progress to establish whether any particular exon combinations are specifically associated with neoplasia.

The most important new finding was that a noncoding section of the gene, intron 9, was clearly detected in mRNA from the fresh colorectal carcinoma tissue specimens (Fig. 4), confirming previous observations on bladder carcinoma biopsies and exfoliated cells. The retention of this intron was seen in mRNAs from 16 (80%) of 20 tumors from which sufficient RNA was available for poly(A)selection. The intensities of the signals and the patterns of hybridization were variable from case to case, but several bands were observed at molecular weight 1.3 kb or higher. This suggests that there are several species of mRNA which contain the intron 9 sequence in combination with various exons and introns. The data could also be indicating that there may be some species in the RNA extracts which are actually in the process of splicing at the time of extraction.

Expression of intron 9 was also detected in four normal samples, although at very low levels compared to tumor tissues (Fig. 4). Our experiments using cell lines, described above, showed that retention of intron 9 occurs in both nuclear and cytoplasmic mRNA fractions.

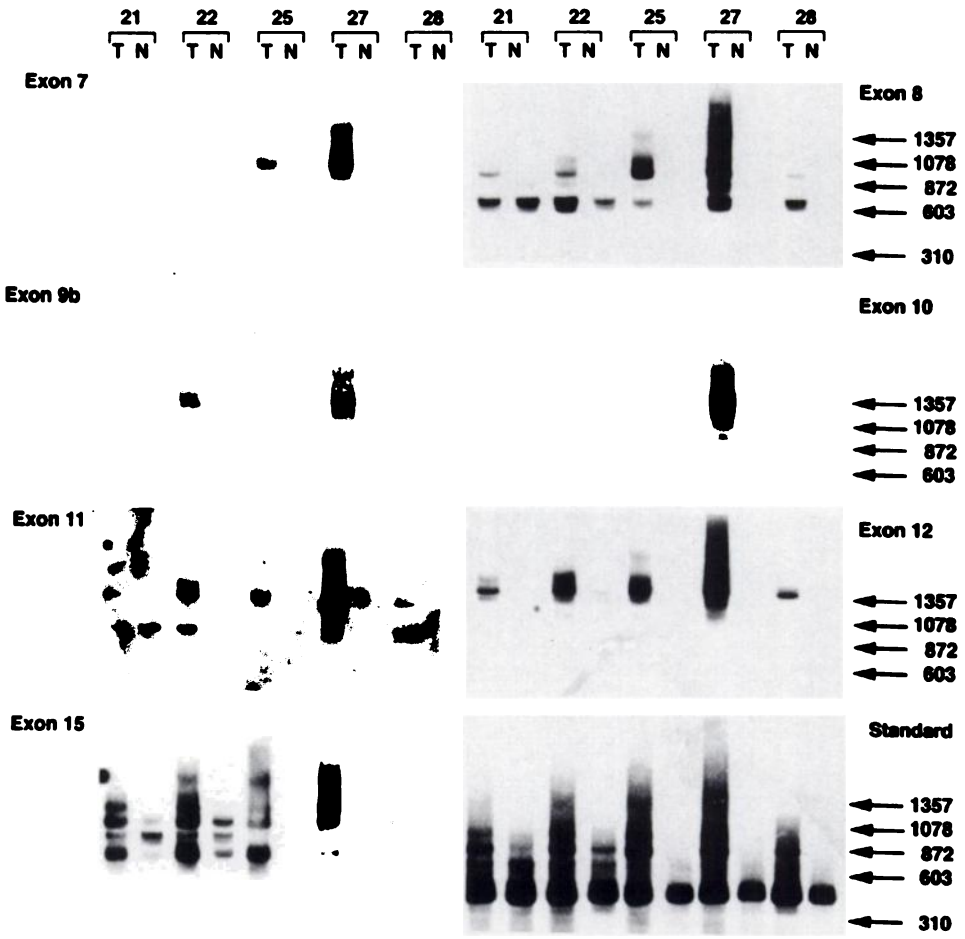

Fig. 3. Southern blot hybridization analysis of RT-PCR products using primers P1, P4, showing the expression of variant exons as labeled. RNA was extracted from human colon carcinoma tissues (*T*) and from matched adjacent normal mucosa (*N*). The numbers above the lanes are case numbers. The filters were stripped and reused for each experiment.

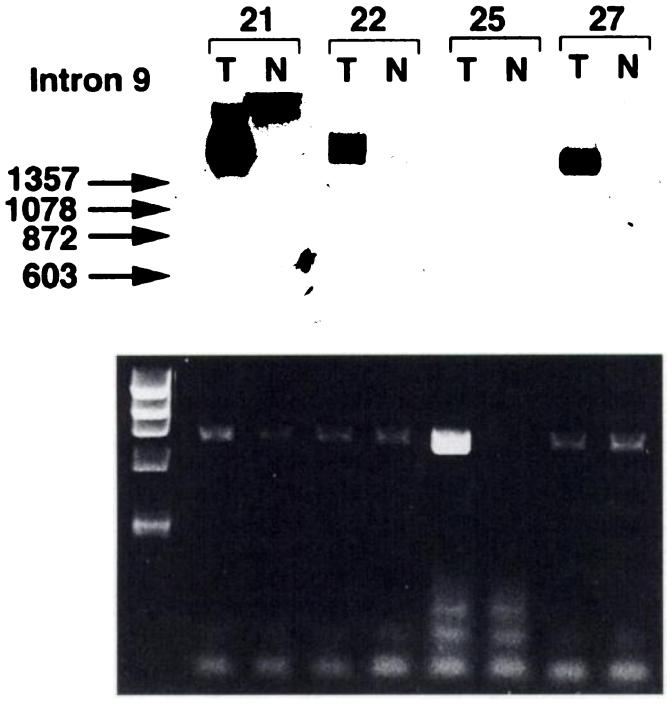

Fig. 4. Demonstration of the abnormal retention of CD44 intron 9 in mRNA transcripts from human colon carcinoma tissues (*T*) and matched adjacent normal mucosa (*N*). One hundred ng of poly(A)$^+$ selected RNA was used for each RT-PCR reaction with E1, E5 primers. The estimated size of these amplicons containing both exon 9a and intron 9 is about 1.6 kb. Ethidium bromide staining of the gel is presented below the Southern blot hybridization autoradiogram. The marker used was Phi X 174 *Hae*III.

Based on this work, our current interpretation is that in normal specimens, we were detecting immature pre-mRNA in the epithelial cell nuclei. However, as the levels of intron 9 retention clearly and reliably discriminate between tumor tissues and normal counterparts, the difference is of potential clinical value.

Although some recent clinicopathological studies have suggested that elevated expression of certain CD44 variants correlates with more rapid progression of disease to metastasis and/or reduced length of survival (6–9), we did not find any correlation between the clinical (Dukes') stage or histological type of these colonic tumors and the retention of intron 9 in their mRNAs. However, the high specificity of detection of early stage bladder cancer using intron 9 retention as a marker for exfoliated cancer cells in the urine (17) may indicate that the disturbances in CD44 gene transcription, which result in the retention of introns, occur early in the neoplastic process and are more useful for early diagnosis than for evaluation of prognosis. Practically, it is conceivable that a non- or minimally invasive method for investigation of patients for colorectal cancer be based upon detection of abnormal CD44 gene expression in cells retrieved from stool samples or from colonic luminal washings. Although the biological significance of the retention of intron 9 in CD44 mRNA is not yet known, the data indicate that it could be a good diagnostic marker for colorectal carcinomas, perhaps in conjunction with abnormal patterns of expression of the variant exons of the gene.

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