Increased Expression of Human Ribosomal Phosphoprotein P0 Messenger RNA in Hepatocellular Carcinoma and Colon Carcinoma

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ABSTRACT

To search for differentially expressed gene products in selected cancers of endodermal origin, cDNA libraries derived from mRNA in human hepatocellular carcinoma and adjacent grossly normal tissue were generated. From these parent libraries, subtracted cDNA libraries of tumor minus normal and normal minus tumor tissues were constructed. After screening these subtracted libraries by $\pm$ hybridization, a cDNA clone that is overexpressed in hepatocellular carcinoma and encodes the human acidic ribosomal phosphoprotein P0 (P0) was identified. We then evaluated the expression of this phosphoprotein P0 in human colon carcinoma samples. Surgical specimens of primary tumors and liver metastases were examined by Northern hybridization of total RNA with one of 23P-labeled P0 probes. The mRNA level of the P0 was greater in primary colon carcinoma than in paired adjacent normal colonic epithelium in 36 of 38 cases; the mean tumor/normal ratio was 2.7 (range, up to 13). The tumor/normal ratio, when plotted against the Dukes' stage of disease, gave evidence for increasing P0 expression with increasing stage of colon carcinoma ($P = 0.02$). In all 8 cases of paired colon carcinoma metastatic to liver and 2 cases of paired primary hepatocellular carcinoma, the P0 mRNA level was greater in tumor than in adjacent normal liver tissue. The mean tumor/normal ratio was 4.0 (range, up to 11) for the colon cancers metastatic to liver and 4.2 for the primary hepatocellular carcinoma samples. These findings suggest a common increased expression of selected gene products in different tumors of endodermal origin and suggest that increased P0 expression, in line with other ribosomal proteins, may be associated with human colorectal cancer progression and biological aggressiveness.

INTRODUCTION

The identification of markers for colorectal carcinoma with increased expression in poorly differentiated, advanced, or metastatic lesions could prove extremely useful in tumor detection or in the estimation of prognosis. Potential new markers from our laboratories include the laminin-binding protein and ubiquitin hybrid protein, whose increased mRNA expression appears to correlate with Dukes' classification of colorectal carcinoma (1, 2). The technique of subtractive cDNA cloning has been used to determine tissue-specific mRNA expression; for example, the isolation of T-cell versus B-cell receptors (3) and the finding of a 10-fold reduced expression of low-abundance preprosomatostatin I mRNA in patients with Alzheimer's disease (4).

The common expression of carcinoma-related antigens in different tissues of endodermal origin, such as liver and colon, has been reported previously (5). Thus, to screen for other common gene products we elected to generate subtractive cDNA libraries from HCC and adjacent normal tissue from the same patient and to screen the differentially expressed clones against mRNA from a series of gastrointestinal tumors and their respective adjacent normal tissues. Recent interest in human ribosomal phosphoproteins has developed for several reasons: (a) their increased expression in specific primary and metastatic tumors (6, 7); (b) the presence of autoantibodies against these proteins in systemic lupus erythematosus (8); and (c) the possibility that these same proteins may function in a dual capacity as DNA repair proteins (9). We report our findings with a cDNA clone isolated with preferential overexpression in HCC tissue, which codes for P0, and which was used to probe samples of primary colon adenocarcinoma, colon adenocarcinoma metastatic to liver, and primary hepatocellular carcinoma.

MATERIALS AND METHODS

Tissue Specimens. Surgical specimens were obtained from the New England Deaconess Hospital Department of Surgery. Thirty-eight pairs of primary human colon carcinomas and adjacent normal colon tissue, 8 pairs of metastatic colon carcinoma to liver and adjacent normal liver, and 3 pairs of primary hepatocellular carcinoma and adjacent grossly normal liver tissue were obtained fresh from the operating room. Necrotic and ulcerated parts of the tumors were removed and normal colonic epithelium was dissociated from muscle and connective tissue as applicable. All tissue samples were then frozen rapidly in liquid nitrogen.

Reverse Transcription-Polymerase Chain Reaction Amplification. Five µg of RNA from each of the HCC and adjacent normal liver samples used to prepare the cDNA libraries were separately converted to cDNA in the presence of HBV-S gene (10) and HCV 5' nontranslated or NS3 sequence (11) downstream oligonucleotide primers. PCR amplification (nested PCR for HCV sequences) was completed in the presence of the relevant upstream primers as detailed (10, 11). Controls utilized RNA from hepatoma cell line CRL 8024, which contains integrated HBV, and from a donor human liver rejected for liver transplantation because of serological markers for HCV. Negative controls omitted the reverse transcriptase enzyme from the incubation. PCR products were separated on agarose gel electrophoresis, transferred to GeneScreenPlus nylon filters (DuPont, Boston, MA) (12), and probed by DNA hybridization with relevant 32P-labeled HBV or HCV probes internal to the PCR primers (10, 11).

Extraction of Total RNA and Northern Blot Hybridization. Total cellular RNA was extracted from surgical specimens or cell lines according to the methods described previously (13), with some modifications and as detailed (1). Equal amounts (15 µg) of total cellular RNA were electrophoresed in 1% agarose-formaldehyde gels. RNA was transferred to GeneScreenPlus nylon filters (DuPont) (14). Only those

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The abbreviations used are: HCC, hepatocellular carcinoma; P0, human acidic ribosomal phosphoprotein P0; ds, double stranded; ss, single stranded; LB, Luria-Bertani medium; HBV, hepatitis B virus; HCV, hepatitis C virus; T, tumor; N, normal; TL, metastatic lesion to liver; NL, adjacent normal liver; TC, primary colon cancer; NC, adjacent normal colon mucosa; PCR, polymerase chain reaction.
filters or samples with visually similar intensities of non-degraded T or N 28S and 18S rRNA under UV illumination were used to calculate T/N ratios. Filters were prehybridized, hybridized, washed, and exposed as outlined (1).

For generation of probes: (a) cDNA inserts were cut from clones of interest using Xbal/HindIII or Xhol/Sacl endonuclease digestion; (b) independently, a full length P0 cDNA clone was isolated from an HT-29 human colon carcinoma cell line cdNA library and was used as a second P0 probe; and (c) a cDNA clone coding a partial sequence of β-actin (15) was used as an internal control as indicated. The inserts were purified by agarose gel electrophoresis, recovered using Geneclean (Bio 101, La Jolla, CA), and labeled with P-32P]-dCTP (20 Ci/mmol) using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Specific activities of the probes were ~2-5 x 10^6 cpm/µg cdNA. Hybridization signals were quantitated by an LKB Ultrascan XL enhanced laser densitometer (LKB Produkter AB, Bromma, Sweden) and analyzed by computing the LKB 2400 GelScan XL software package. The integrated area under the density curves produced the tumor/normal ratios as an estimate of mRNA expression, although the signal density may not be fully linearly related to mRNA expression.

cDNA Library Construction. Selection of polyadenylated mRNA from total cellular RNA was based on methods described previously (16) or utilized the Fast-track mRNA selection kit (Invitrogen, San Diego, CA). A plasmid cdNA library construction system (Librarian II; Invitrogen) was used, with modifications, to generate cdNA libraries from hepatocellular carcinoma and adjacent grossly normal tissues from the same patient. Double stranded cdNAs were synthesized by published methods (17) from 2-5 n% poly(A)* RNA with Oligo(dT) priming. Nonpalindromic BST XI cut linkers were ligated to the blunt ended ds cdNA. Unligated linkers and ds cdNA of size <400 nucleotides were removed by size selection through a Sephacryl S-400 exclusion column (Pharmacia, Piscataway, NJ). The sized ds cdNA was ligated to pre-prepared BST XI cut vector pcdNA II. Ligation mixtures were transformed into, freshly thawed Escherichia coli INVµF'. The cdNA library was stored in 15% glycerol at —70°C, and aliquots were plated onto LB medium with 50 µg/ml ampicillin, 1.5% agar, containing 1 mM isopropyl l-thio-0-D-galactoside (BRL, Gaithersburg, MD) and 0.1% 5-bromo-4-chloro-3-indoyl-β-D-galactoside (BRL).

Subtraction Libraries. Plasmid cdNA libraries were prepared as outlined above from: (a) HCC tissue; and (b) adjacent grossly normal liver from the same patient. Two subtraction libraries, HCC minus normal and normal minus HCC, were prepared using a subtraction cdNA library construction system (Invitrogen) with modifications. The parent plasmid cdNA libraries were amplified on solid medium (LB with 50 µg/ml ampicillin) and then converted to cdNA. Twenty µg ss cdNA from either the HCC or normal liver source were irradiated for 15 min under a sun lamp (GE/RSM, 275 W) in the presence of photobiotin acetate. The photobiotinylolation process was repeated to increase the efficiency of cross-linking; 2.5 µg of ss cdNA from the alternate HCC or normal liver tissue source were ethanol-pelletized in the presence of an approximately 8-fold m excess of the photobiotinylated cdNA and, after heating to 100°C for 1 min, hybridization was continued at 68°C for 16-20 h. Photobiotinylated cdNA and hybridized fragments were selectively precipitated with streptavidin (1 mg/ml) and then end-labeled with γ-32P]-ATP and T4 polynucleotide kinase according to details outlined in the subtractive cDNA probe kit (Invitrogen) to yield probes of specific activity.

Library Screening: ± Hybridization. Aliquots of the subtracted library were grown on LB medium with 50 µg/ml ampicillin, 1 mM isopropyl l-thio-β-D-galactoside, and 0.1% 5-bromo-4-chloro-3-indoyl-β-D-galactoside. Selected white colonies with inserts were transferred in triplicate to Duralose-UV membranes (Stratagene) in a grid pattern for ± hybridization using ±-labeled cdNA probes prepared from the original tumor and normal sources of cdNA. Probed ± pairs of filters were compared and differentially expressed clones replated from the master and reprobed to verify selective expression.

Clinicopathological Data. Age, sex, tumor location, histology, tumor size, carcinoembryonic antigen level, pathological data relating to depth of invasion, lymph node metastasis, liver metastasis, and follow-up data were obtained from the hospital records of each patient. The Dukes' staging (18, 19) of the primary tumors was: Dukes' A (tumor invading into, but not through the bowel wall); Dukes' B (tumor invading through the bowel wall, without lymph node involvement); Dukes' C (with involvement of regional lymph nodes); and Dukes' D (with distant metastasis).

Statistical Analysis. For data with n > 30, a standard normal distribution was used. For n < 12, Wilcoxon's signed rank statistic was used to determine significance levels. To examine for a trend towards higher expression in different Dukes' stage of disease, an analysis of covariance was used. A P value of 0.025 was required to perform separate tests for trend for stages A-D and stages A-D and liver metastasis.

RESULTS
cDNA Library Generation and Isolation of Clones Encoding P0. Both HCC minus normal and the complementary normal minus HCC subtraction cdNA libraries representing overexpressed and underexpressed gene products were prepared, with a view to the inclusion of potential oncogene products and tumor suppressor gene products, respectively. Parent cdNA plasmid libraries in E. coli INVµF' were prepared from cdNA isolated from samples of human hepatocellular carcinoma and adjacent grossly normal tissue of the same patient. The unamplified libraries contained about 4 x 10^9-2 x 10^10 colonies. After amplification, subtraction cdNA libraries were prepared using ss cdNA and 2 cycles of subtractive hybridization with an 8-fold m excess of the photobiotinylated "undesired" source of DNA. Initial screening of about 2200 colonies with inserts by ± hybridization yielded 33 clones coding for 17 distinct sequences, which had consistent T > N or N > T expression upon repeated plating and hybridization. One of these clones had 100% homology over an 873-nucleotide overlap at the 3'-end of the cdNA of human acidic ribosomal phosphoprotein P0 (20), and lacked the 5'-terminal 151 nucleotides from the initiation codon and the 77 nucleotide 5'-untranslated region. Independently, a full-length P0 cdNA clone was isolated from an HT-29 colon carcinoma cell line cdNA library. Inserts from these 2 P0 cdNA clones were used as probes for the Northern RNA hybridizations. There were no mutational changes in the cdNA sequence from the clones isolated from either the HT-29 cell line or from the hepatoma library that might have contributed to the altered P0 expression.

HBV and HCV may be important in the etiology of HCC (21, 22); we therefore checked the samples of HCC and adjacent normal liver used to prepare the cdNA libraries for any evidence of HBV or HCV sequences by reverse transcription-polymerase chain reaction and Southern hybridization and...
found no evidence for these hepatitis viruses in these samples. Furthermore, no hepatitis-related sequences were isolated from the subtraction and screening process.

Northern Blot Analysis of Primary Colon Carcinoma. Fig. 1 illustrates a representative autoradiogram from a Northern RNA blot, containing 8 paired total RNA samples from patients with primary lesions of colon carcinoma and adjacent normal mucosa probed first with $^{32}$P-labeled PO derived from the HT-29 colon carcinoma cell line and then with $^{32}$P-labeled cDNA encoding a portion of the $\beta$-actin gene. Each individual pair of T/N tissues was derived from the same patient. No difference was noted whether the PO probe was derived from the HCC T/N tissues was derived from the same patient. No difference in the quoted levels of significance was observed with any confounding interpatient variability. For this patient, the T/C ratio of the primary colon carcinoma was 2.2, the TL/NC ratio for the metastatic lesion was 5.0, the metastatic and primary lesions had similar intensities with a TL/TC of 1.1, and the normal colon had a somewhat higher signal than the normal liver, NC/NL = 1.7. Two other patients had simultaneous resections of hepatic metastases and primary colon cancer lesions yielding 3 samples (TL, TC, and NC). Both primary and metastatic colon tumors showed higher levels of PO mRNA than did normal colon mucosa in these 2 patients (Fig. 6B). No consistent difference was noted in the PO mRNA levels between the primary tumor and the respective liver metastases: in one case, the signal from the metastatic lesion was stronger (patient 2, TL > TC); in the other, the primary was stronger (patient 3, TC > TL) and, as mentioned, the patient of Fig. 6A had TL = TC. To facilitate further comparison between T/N hybridization signals from primary colorectal lesions and metastatic lesions, a comparison was made between unpaired normal colon mucosa (n = 4) and unpaired normal liver tissue (n = 5). Comparing ranked or mean signal intensities gave a NC/NL ratio of 1.3 (data not shown).

Fig. 2 includes the T/N hybridization signal density of the autoradiogram for the liver tumors probed with PO. The mean signal density of the PO mRNA from paired samples of primary colorectal carcinoma is shown in Fig. 3, with a maximum of 13 and median 2.2. To investigate whether the increased expression of PO mRNA in colon carcinoma correlated with the extent of disease, the T/N ratio of PO expression for paired samples of primary colon cancer was plotted against the Dukes' stage of disease for the 38 samples (Fig. 4). A trend of increasing PO expression with increasing stage of colon cancer was observed. Analysis of covariance gave evidence for a significant effect for stage ($P = 0.02$ analyzing stages A-D, $P = 0.001$ analyzing stages A-D and liver metastases).

Clinicopathological Data. The study cohort for the colon carcinoma patients presented here consisted of 46 patients, 21 males, and 25 females. The mean age was 69 years (range, 41–87 years). The distribution of primary colon tumors was as follows: cecum, 11; “right” colon, 4; transverse colon, 4; sigmoid colon, 15; rectosigmoid, 3, rectal, 1. Histologically, 40 tumors were classified as moderately differentiated adenocarcinomas; there were one well differentiated and 5 poorly differentiated adenocarcinomas. Samples were classified as Dukes' A, n = 5; Dukes' B, n = 13; Dukes' C, n = 9; and Dukes' D, n = 11. Liver metastasis was present in 8 patients. Median carcinoembryonic antigen level, available for n = 5 Dukes' A patients was 2 (range, 1–27); n = 8 Dukes' B patients was 3 (range, 1–122); n = 7 Dukes' C patients was 7 (range, 1–98); n = 9 Dukes' D patients was 108 (range, 3–4944). The mean (and median) follow-up for n = 44 patients with more than 1 month follow-up was 18 months (range, 2–47 months). The disease status at last follow-up was alive and disease-free, 25; alive with disease, 10; and expired or expired with disease, 11. There was no correlation of PO mRNA expression with age, sex, tumor location, tumor size, or carcinoembryonic antigen level. No conclusion could be made with respect to the differentiation because the majority of specimens was moderately differentiated. There was no change in the quoted levels of significance when the extent of disease was staged by the tumor-node-metastasis system.

Northern Blot Analysis of Hepatic Tumors. Hepatic tumors accounted for a total of 10 samples. Eight were paired metastatic colon carcinomas and 2 were primary HCC; all of these 10 had T/N ratios >1.0 for the PO hybridization signals. Two HCC cell lines (HB 8065 and CRL 8024) had signals greater than any normal (or tumor) liver tissue values for the same amount of applied total RNA (data not shown). Fig. 5 illustrates an autoradiogram from a Northern blot of RNA from patients with primary hepatocellular carcinoma (Fig. 5A, n = 2) and colon carcinoma metastatic to liver (Fig. 5B, n = 3). A single patient had 4 simultaneous tissues resected (Fig. 6A): TL, NL, TC, and NC. This provided the opportunity to compare the level of PO mRNA expression, between liver and colon, without any confounding interpatient variability. For this patient, the TC/NC ratio of the primary colon carcinoma was 2.2, the TL/NC ratio for the metastatic lesion was 5.0, the metastatic and primary lesions had similar intensities with a TL/TC of 1.1, and the normal colon had a somewhat higher signal than the normal liver, NC/NL = 1.7. Two other patients had simultaneous resections of hepatic metastases and primary colon cancer lesions yielding 3 samples (TL, TC, and NC). Both primary and metastatic colon tumors showed higher levels of PO mRNA than did normal colon mucosa in these 2 patients (Fig. 6B). No consistent difference was noted in the PO mRNA levels between the primary tumor and the respective liver metastases: in one case, the signal from the metastatic lesion was stronger (patient 2, TL > TC); in the other, the primary was stronger (patient 3, TC > TL) and, as mentioned, the patient of Fig. 6A had TL = TC. To facilitate further comparison between T/N hybridization signals from primary colorectal lesions and metastatic lesions, a comparison was made between unpaired normal colon mucosa (n = 4) and unpaired normal liver tissue (n = 5). Comparing ranked or mean signal intensities gave a NC/NL ratio of 1.27 (data not shown).

Fig. 1. Northern blot analysis of RNA from paired samples of primary colon carcinoma and adjacent normal colonic epithelium probed with $^{32}$P-labeled cDNA encoding human ribosomal phosphoprotein PO (top panel), and then with $^{32}$P-labeled cDNA encoding $\beta$-actin (middle panel). Bottom panel, agarose gel under UV illumination with positions of 28S rRNA and 18S rRNA indicated. n = 8 pairs; patient 7 has degraded RNA.
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Fig. 2. Results of Northern blot analysis of RNA from carcinoma and paired normal samples probed with 32P-labeled cDNA encoding human ribosomal phosphoprotein PO. Tumor/normal ratio of hybridization signal density is expressed as mean ± SE (except for HCC ± range). Ho, null hypothesis that tumor = normal; PRIMARY COLON CA, paired samples of primary colon carcinoma and adjacent normal mucosa (n = 38); HCC, paired samples of HCC and adjacent normal mucosa (n = 38); HCC, paired samples of HCC and adjacent normal liver (n = 2); L-MET, paired samples of colon carcinoma metastatic to the liver and adjacent normal liver (n - 8); TOTAL combines the T/N ratios for all the tumors (n = 48).

Fig. 3. Results of Northern blot analysis of RNA from primary colon carcinoma and paired normal colon samples probed with 32P-labeled cDNA encoding human ribosomal phosphoprotein PO. The number of tumor pairs is plotted against the range of tumor/normal ratios of hybridization signal density for paired samples (n = 38), each pair from the same patient.

T/N ratio for the metastatic colon carcinoma samples (n = 8) was 4.03, and for all of the hepatic tumors it was 4.06, significantly different from a ratio of 1.0 (P = 0.001). The 2 HCC samples had a mean T/N ratio of 4.2. The distribution of T/N ratios for the hepatic tumors had a maximum of 10.8 and median of 2.1.

To summarize, a total of 48 carcinoma tissue samples and corresponding paired normal samples were analyzed. Probing Northern blots with 32P-labeled PO yielded an autoradiography signal from T tissue greater than or equal to N in 46 of 48 cases (96%). The signals were consistent with a transcript size of 1.2–1.6 kilobases. Of the paired primary colon carcinomas, 87% had a T/N ratio >1.2, 10.5% had an approximately equal ratio (defined as T/N 0.8–1.2), and 2.5% had a signal less dense than the tumor (defined as T/N < 0.8). All of the hepatic tumors had a T/N ratio >1.2.

DISCUSSION

There is evidence for the dual expression of carcinoma-related antigens in tissues of endodermal origin, such as liver and colon. For example, a monoclonal antibody (SF-25) raised against a human hepatoma cell line (FOCUS) detected a Mr 125,000 cell surface antigen in 23 of 23 colon adenocarcinoma tissues, but not in the adjacent normal mucosa (5). We thus screened differentially expressed clones, isolated by subtractive cDNA hybridization of hepatocellular carcinoma versus normal tissue, against a series of colorectal carcinoma tissues. One of the selectively overexpressed clones in HCC was found to encode the ribosomal phosphoprotein PO, which shares a highly conserved C-terminal sequence with P1/P2, which are believed to interact with eukaryotic initiation, elongation, and releasing factors (23–25). P0 may be analogous to the prokaryotic L10 protein (20). The level of PO mRNA expression was increased in the majority of tumors tested. To reduce the effect of the inherent variability in expression between patients, only T/N ratios (calculated with both T and N samples from the same patient) were utilized in the analysis.

P-proteins may be readily able to exchange on/off the ribosome (26). This may contribute to the formation of autoantibodies to P-proteins in some patients with systemic lupus erythematosus (8, 27). This raises the possibility that autoantibodies may be generated against overexpressed P-proteins in patients with colorectal cancers. However, assays for IgM/IgG P-protein antibodies in the serum of 11 patients with Dukes' stage D colon carcinoma were negative. The use of antibodies against P-proteins may permit evaluation of the expression of this gene product, at the protein level, on tumor cells from archival specimens and an attempt to correlate the data with survival statistics. The ready removal of the P-proteins from ribosomes also may be relevant in their proposed role as dual function proteins. There is significant homology (65% in 317 amino acid overlap) between human PO and Drosophila repair gene AP3 (9). Thus, it is possible that P0 has a function both as an integral ribosomal protein, and additionally as a DNA repair protein free of ribosomal attachment. Phosphorylation of the P-proteins may increase their affinity for the ribosome (28). The proposed role as dual function protein/enzyme is attractive for a protein with the capacity to be phosphorylated, since there are many examples of phosphorylation of tyrosine, serine, and threonine residues affecting regulatory processes (29). Ribosomal protein S6 is phosphorylated during development, tissue regeneration, growth, and transformation, in part by tyrosine-specific kinases (for review, see Ref. 30). Therefore, it will be of considerable interest to further define the nature and role of the phosphorylation status and corresponding structural/enzymic activities of the P-proteins, particularly P0.

With respect to ribosomal protein expression in neoplastic tissues, increased expression of L31 mRNA in 23 of 23 colo-
rectal tumors was noted (31). After submission of this paper, ribosomal protein S3 mRNA was reported to be increased in colon cancer (32). Additionally, P2 had enhanced mRNA expression in liver metastases and in primary colon carcinoma compared to normal colonic mucosa (n = 3) (6, 7). Contrary to expectations, it was also increased ~5-fold in breast fibroadenomas compared to breast carcinomas (7). Thus, for P2 at least, the increased P-protein mRNA expression was not specific for cancer. We did not assess the level of P0 mRNA in other nonmalignant colonic diseases (such as diverticulitis, inflammatory bowel disease, or adenomas), and therefore cannot exclude the possibility that increased P0 expression may occur in benign conditions. However, we have noted no increased P0 mRNA expression in gastric carcinoma samples, thus the elevation is not a uniform finding in all cancers, nor even all gastrointestinal cancers. In our laboratory, we have noted increased expression in human colorectal cancer of some other ribosomal proteins including S6 (33) and the ubiquitin-S27a hybrid protein that correlated with Dukes’ stage of disease (2). Not all studied ribosomal proteins were increased. The basis for the increased expression of P0 observed in this study is not readily apparent. It is possible that protein synthesis is highly activated during progression and metastases of colon tumors, and that these selected ribosomal proteins, particularly those such as P0, whose expression correlates with Dukes’ stage of disease, may prove useful as markers of biological aggressiveness in colorectal carcinoma.

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