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Characterization of Cytokeratin 20 Expression in Pancreatic and Colorectal Cancer[1]

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ABSTRACT

Cytokeratin 20 belongs to the epithelial subgroup of the intermediate filament family. Because of its restricted range of expression in humans, it has become an important tool for detecting and identifying metastatic cancer cells by immunohistochemistry and by PCR analysis. Despite its widespread diagnostic use in colorectal cancer and occasional use in pancreatic cancer, little is known about the expression of CK 20 in these tumors *in vivo*. Therefore, in the present study we characterized CK 20 expression in pancreatic and colorectal cancer by comparison with its expression in the normal pancreas and colon. Tissue samples from 24 patients with pancreatic cancer and from 41 patients with colorectal cancer were examined for CK 20 expression by Northern blot analysis, immunohistochemistry, and *in situ* hybridization. CK 20 expression was observed in the cancer cells of both cancer types. A subgroup of the pancreatic cancers exhibited a 3.2-fold increase in CK 20 mRNA by comparison with respective normal controls. In contrast, colon cancers underexpressed CK 20 mRNA by comparison with the respective controls. In the normal tissues, CK 20 immunoreactivity was relatively faint and sparse in the pancreatic ductal cells but intense and abundant in the apical portions of the colonic mucosa. CK 20 immunoreactivity was also evident in the ductal cells from the chronic pancreatitis-like lesions adjacent to the cancer cells. Furthermore, distant metastases from pancreas carcinomas exhibited strong CK 20 immunoreactivity. It is concluded that CK 20 is overexpressed in pancreatic cancer and that it can serve as an excellent marker for metastatic pancreatic cancer.

INTRODUCTION

CKs[3] are a family of proteins that form the intermediate filament cytoskeleton of epithelial cells. They consist of two different groups, the type I acidic keratins and the type II basic keratins (1). The first step in keratin intermediate filament assembly is the formation of heterodimers between a type I and a type II keratin (2, 3). To date, 20 distinct polypeptides, which belong to this family, have been identified in various human epithelial cell types (4–7). The differential expression of these proteins is closely linked with specific programs of differentiation (8–11). Thus, cytokeratin expression correlates with different epithelial cell lineages, allowing for the classification of epithelial cells into numerous subtypes (12–16). The most recently described member of this family is the 46-kb cytoskeletal protein CK 20.

CK 20 is consistently detected in human intestinal mucosa, urothelium, and Merkel cells (17). It is expressed preferentially in differentiated enterocytes and goblet cells of the intestinal villi (17). In contrast, in crypts, only a few of the undifferentiated cells are CK 20 positive (17). CK 20 has been detected in the pancreata of rat and pig (18, 19). In the rat, CK 20 expression was restricted to the duct cells (19). In addition, CK 20 may be considered as a "maturation" marker of the biliary tree in the liver of the rat (20). There is one report of CK 20 immunoreactivity in primary pancreatic cancers (21) and a recent report of its presence in pancreatic cancer metastases to the liver (22). The expression of several other cytokeratins (CK 7, CK 8, CK 18, and CK 19) has also been reported in the human pancreas (18). Because of the lack of immunological cross-reactivity with other cytokeratins, CK 20 has become an important tool for delineating the origin of metastatic human adenocarcinomas arising from an unknown primary source (23–33). In addition, several reports have highlighted the importance of detecting CK 20 by the use of the PCR as a marker for the spread of tumor cells into blood, bone marrow, and lymph nodes of colorectal and other gastrointestinal cancers such as gastric and pancreatic cancers (34–40).

Colorectal cancer is the second most common cause of cancer death and the fourth most prevalent carcinoma in the Western world. In the United States, there were ~131,000 new cases of colorectal cancer and 55,000 deaths from this disease in 1997 (41). Similarly, pancreatic cancer is a devastating disease

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[3] The abbreviation used is: CK, cytokeratin.

that is the fifth most common gastrointestinal tract cancer in males and the seventh most common in females. Its high mortality rate makes it the fourth most common cause of death from cancer in the general population. To date, the level of expression of CK 20 in either colon or pancreatic cancer by comparison with the respective normal tissues has not been reported. Furthermore, the exact site of CK 20 expression in either malignancy has not been described. Therefore, in this study we compared the expression of CK 20 in human colorectal and pancreatic cancer with its expression in the corresponding normal tissues. We now report that CK 20 is expressed at high levels in a subgroup of pancreatic cancers but not in colon cancers, and that CK 20 expression in pancreatic cancer occurs predominantly in the cancer cells.

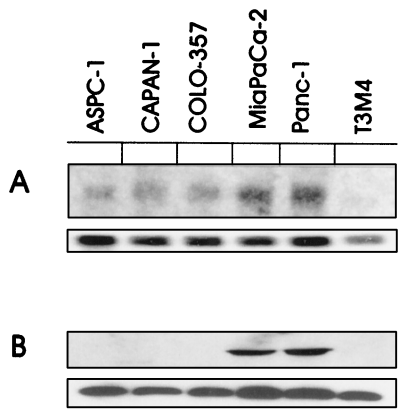

Fig. 1 Expression of CK 20 in cultured pancreatic cancer cells. *A*, Northern blotting. Total RNA (20 μg/lane) was prepared from the indicated cell lines and hybridized with ^{32}P-labeled cDNA probes (500,000 cpm/ml) specific for CK 20. A 7S ribosomal cDNA probe (50,000 cpm/ml) was used as a loading and transfer control. Exposure time was 12 h for CK 20 and 4 h for 7S. *B*, Western blotting. Lysates from six pancreatic cancer cell lines were probed with a highly specific CK 20 antibody (see "Materials and Methods"). Immunoblotting with anti-ERK2 antibodies was used as a loading and transfer control. Exposure times were 2 min for CK 20 and 1 min for ERK2.

MATERIALS AND METHODS

Materials. The following materials were purchased: fetal bovine serum, DMEM, RPMI, Leibovitz L-15, trypsin solution, and penicillin-streptomycin solution from Irvine Scientific (Santa Ana, CA); restriction enzymes and the random primed labeling kit from Boehringer-Mannheim (Indianapolis, IN); Sequenase Version 2.0 DNA Sequencing from United States Biochemical Corp. (Cleveland, OH); [α-^{32}P]dCTP, [γ-^{35}S]dATP from Amersham (Arlington Heights, IL); and pBluescript-IISK+ from Stratagene (La Jolla, CA). A highly specific monoclonal mouse anti-human CK 20 antibody was from Dako (Carpinteria, CA). All other chemicals and reagents were purchased from Sigma Chemical Corp. (St. Louis, MO). ASPC-1, Capan-1, Panc-1, and Mia-PaCa-2 human pancreatic cell lines as well as CaCo2, SW 837, and SW 1463 human colorectal cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). COLO-357 and T3M4 human pancreatic cancer cell lines were a gift from Dr. R. S. Metzger (Durham, NC).

Tissue Samples. Normal human pancreatic tissue samples (n = 18) were obtained through an organ donor program. Pancreatic cancer tissues (n = 24) were surgical specimens obtained from pancreatic cancer patients. Normal colon tissue samples (n = 41) as well as cancer samples (n = 41) were obtained from patients undergoing colorectal cancer surgery. The normal tissue was taken from the same patients at a distance of at least of 10 cm from the tumor site. According to the Tumor-Node-Metastasis classification of the Union Internationale Contre le Cancer, there were 9 stage I, 3 stage II, and 12 stage III pancreatic cancers and 13 stage I, 7 stage II, 7 stage III, and 14 stage IV colorectal cancers. Freshly removed tissue samples were fixed in Bouin or 10% formaldehyde solution and paraffin-embedded for histological analysis. In addition, tissue samples were frozen in liquid nitrogen immediately upon surgical removal and stored at −80°C until use for RNA extraction. All studies were approved by the Ethics Committee of the University of Bern and by the Human Subjects Committee of the University of California, Irvine.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted by the single-step acid guanidinium thiocyanate-phenol-chloroform method (42). RNA was size fractionated on 1.2% agarose/1.8 M formaldehyde gels, electrotransferred onto nylon membranes, and cross-linked by UV irradiation. Blots were prehybridized and hybridized with CK 20 cDNA probe and washed under high stringency conditions as reported previously (43). Blots were then exposed at −80°C to Kodak BiomaxMS films, and the resulting autoradiographs were scanned to quantify the intensity of the radiographic bands. A *Bam*HI 190-kb fragment of mouse 7S cDNA that hybridizes with human cytoplasmatic RNA was used to confirm equal RNA loading and transfer (43).

Immunoblotting. Cells were washed with PBS (4°C) and solubilized in Tris-buffered saline (TBS) containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM EGTA, 2.5 mM $NaPO_4$, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM β-glycerophosphate, and 10 μg/ml leupeptin. Proteins were subjected to SDS-PAGE and transferred to Immobilon P membranes. Membranes were incubated for 60 min with anti-CK 20 antibodies, washed, and incubated with a secondary antibody against mouse for 60 min. After washing, visualization was performed by enhanced chemiluminescence. Membranes were then washed twice with TBS containing 0.1% Tween 20 (TTBS) for 30 min and reprobed with anti-ERK2 antibody to confirm equivalent loading of lanes.

Immunohistochemistry. A highly specific, affinity-purified mouse monoclonal antibody to human CK 20 was used for immunohistochemistry (11). Paraffin-embedded sections (4 μm) were subjected to immunostaining using the streptavidin-peroxidase technique. Sections then were boiled for 5 min in 10 mM citrate buffer. Endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in methanol. Tissue sections were incubated for 15 min (room temperature) with 10% normal goat serum and then incubated for 16 h at 4°C with CK 20 antibody (5 ng/ml) in PBS containing 1% BSA. Bound antibodies were detected with biotinylated goat anti-mouse IgG secondary antibodies and streptavidin-

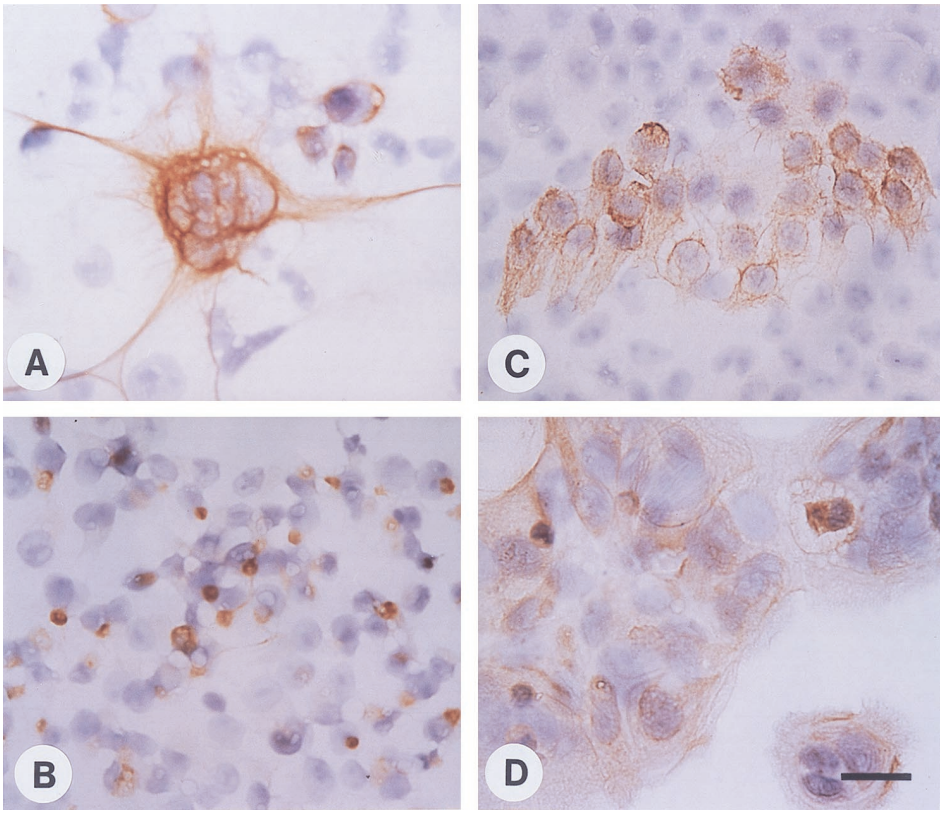

Fig. 2 CK 20 immunoreactivity in cultured cancer cell lines. Moderate to strong CK 20 immunostaining was visible in MiaPaCa-2 (*A*) and Panc-1 (*B*) pancreatic cancer cells and in small foci of COLO-357 pancreatic cancer cells (*C*). SW 1463 colorectal cancer cells also exhibited CK 20 immunoreactivity (*D*). *Bar*, 25 μm.

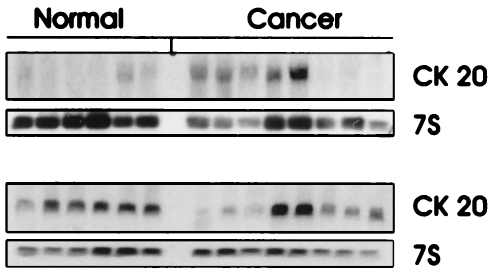

Fig. 3 Expression of CK 20 mRNA in pancreatic tissues by Northern blotting. Total RNA (20 μg/lane) was prepared from pancreatic tissues and hybridized with ^{32}P-labeled cDNA probes (500,000 cpm/ml) specific for CK 20. A 7S ribosomal cDNA probe (50,000 cpm/ml) was used as a loading and transfer control. Exposure times were 12 h for CK 20 and 4 h for 7S.

peroxidase complex, using diaminobenzidine tetrahydrochloride as the substrate. Sections were counterstained with Mayer's hematoxylin. Sections incubated without primary antibody did not yield positive immunoreactivity.

***In Situ* Hybridization.** To carry out *in situ* hybridization analysis, tissue sections (4 μm thick) were placed on 3-aminopropyl-methoxysilane-coated slides, deparaffinized, and incubated at 23°C for 20 min with 0.2 N HCl and at 37°C for 15 min with 40 μg/ml (10 μg/ml for colon) proteinase K. The sections were then postfixed for 5 min in PBS containing 4% paraformaldehyde and incubated briefly twice with PBS containing 2 mg/ml glycine and once in 50% (v/v) formamide/2× SSC (1× SSC = 150 mM NaCl, 15 mM nacitrate, pH 7.0) for 1 h prior to initiation of the hybridization reaction by the addition of 100 μl of hybridization buffer. The hybridization buffer contained 0.6 M NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.6), 0.25% SDS, 200 μg/ml yeast tRNA, 1× Denhart's solution, 10% dextran sulfate, 40% formamide, and the digoxigenin-labeled riboprobe. The CK 20 was labeled with digoxgenin-UTP by T3 or T7 RNA polymerase using the Genius 4 RNA labeling kit. Hybridization was performed in a moist chamber for 16 h at 50°C. The sections were then washed sequentially with 50% formamide/2× SSC for 20 min at 42°C, 2× SCC for 20 min at 42°C, and 0.2× SSC for 20 min at 42°C. The Genius 3 nonradioactive nucleic acid detection kit was used for immunological detection. The sections were washed briefly with buffer 1 solution (100 mM Tris-HCl and 150 mM NaCl, pH 7.5) and incubated with 1% (w/v) blocking reagents in buffer 1 solution for 60 min at 23°C. The sections then were incubated for 30 min at 23°C with a 1:2000 dilution of an alkaline phosphatase-conjugated polyclonal sheep anti-digoxigenin Fab fragment antibody, washed twice for 15 min at 23°C with buffer 1 solution containing 0.2% Tween 20, and equilibrated with buffer 3 solution (100 mM Tris-HCl, 100 mM NaCl, and 50 mM $MgCl_2$, pH 9.5) for 2 min. The sections were then incubated with color solution containing nitroblue tetrazolium and X-phosphate in a dark box for 2–3 h. After the reaction was stopped with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), the sections were mounted in aqueous mounting medium.

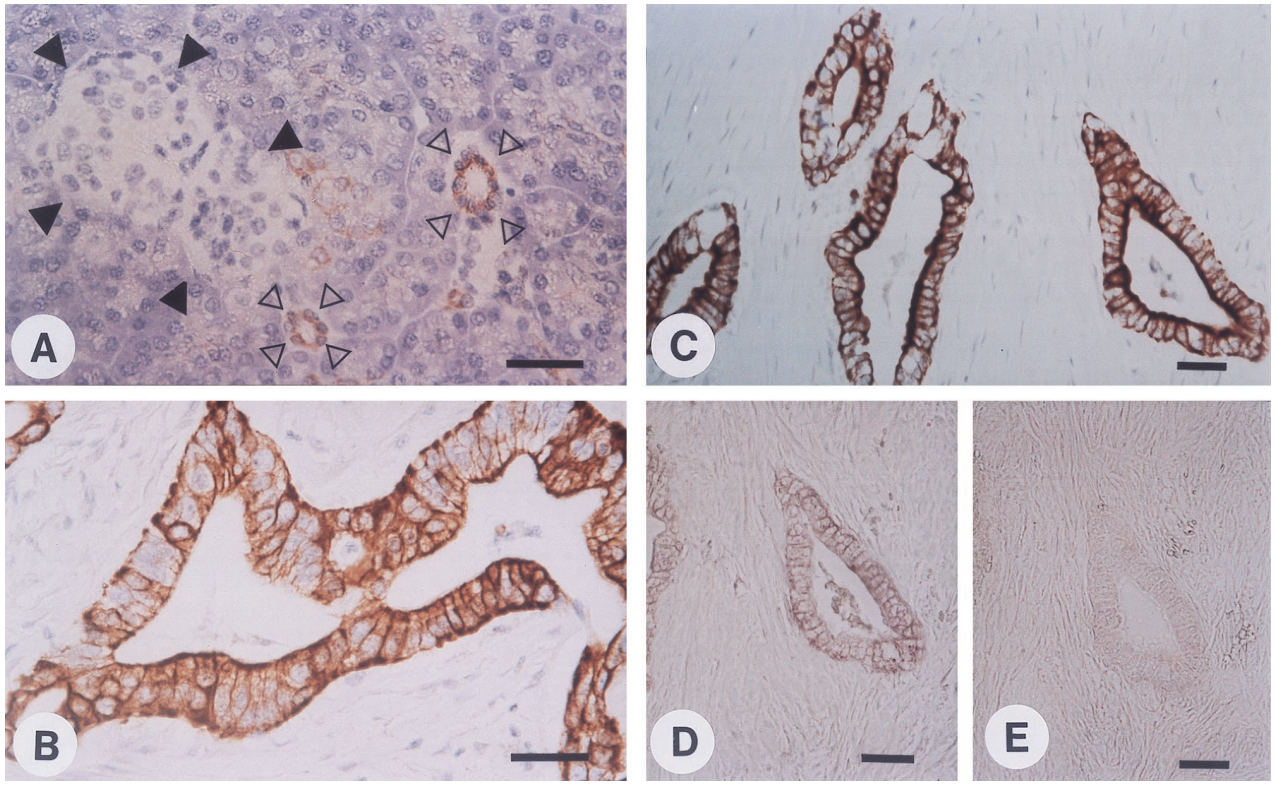

Fig. 4 Comparison of immunohistochemistry and *in situ* hybridization in pancreatic cancer tissues. There was faint CK 20 immunoreactivity in the ductal cells in the normal pancreas (*A, open arrowheads*), which was not seen in either the endocrine islets (*A*, outlined by *closed arrowheads*) or acinar cells. In contrast, intense CK 20 immunoreactivity was present in the pancreatic cancer cells within the tumor mass (*B* and *C*). Analysis of serial sections revealed that cancer cells exhibiting CK 20 immunoreactivity (*C*) also exhibited a moderate to strong CK 20 mRNA *in situ* hybridization signal (*D*). *In situ* hybridization with the CK 20 sense probe did not reveal any specific signal (*E*). *Bar*, 25 μm.

RESULTS

CK 20 mRNA Expression in Pancreatic Cell Lines and Tissues. Northern blot analysis of total RNA isolated from six pancreatic cancer cell lines revealed a moderate CK 20 mRNA signal (1.8 kb) in MiaPaCa-2 and Panc-1 cells and a weak signal in ASPC-1, Capan-1, and Colo-357 cells (Fig. 1*A*). In contrast, the CK 20 mRNA transcript was below the level of detection in T3M4 pancreatic cancer cells (Fig. 1*A*). Western blot analysis revealed a distinct band (M_r ~46,000), corresponding to CK 20 in Panc-1 and MiaPaCa-2 cancer cells, whereas this band was not detected in T3M4, COLO-357, Capan-1, or ASPC-1 cells (Fig. 1*B*). Immunohistochemical analysis of the cell lines revealed strong CK 20 immunoreactivity in many MiaPaCa-2 (Fig. 2*A*) and Panc-1 (Fig. 2*B*) cells. In contrast, in COLO-357 cells, there were foci of cells that were CK 20 positive, whereas many other cells were negative (Fig. 2*C*). T3M4 cells were completely devoid of CK 20 immunostaining (not shown). Thus, there was good concordance between the presence of high levels of CK 20 mRNA and protein as detected by immunoblotting when the majority of the cells expressed CK 20. In contrast, immunostaining was even more sensitive than immunoblotting, because it confirmed the presence of CK 20 in a subpopulation of COLO-357 cells.

Northern blot analysis of total RNA isolated from the normal human pancreas revealed that the CK 20 mRNA moiety was present at weak to moderate levels in 11 of 18 samples. Some cancer samples also exhibited weak CK 20 mRNA levels. However, 11 of 24 pancreatic cancer tissues showed moderate to high levels of CK 20 mRNA. Two examples of these Northern blots are shown in Fig. 3. Densitometric analysis of all of the Northern blots revealed a 1.8-fold increase in CK 20 mRNA levels in pancreatic cancer samples in comparison with the normal samples. However, when the results from the 11 samples that overexpressed CK 20 were calculated, there was a 3.2-fold increase ($P = 0.01$) in CK 20 mRNA levels.

Immunohistochemical analysis with the same highly specific anti-CK 20 antibody used above revealed that the ductal cells in the small ducts in the normal pancreas showed faint to moderate CK 20 immunoreactivity (Fig. 4*A*). In contrast, the islet and acinar cells were devoid of CK 20 immunoreactivity (Fig. 4*A*). In the cancer tissues, there was a very strong CK 20 immunoreactivity in the ductal-like cancer cells (Fig. 4, *B* and *C*). A faint CK 20 mRNA signal was detectable by *in situ* hybridization in one of four samples in the ductal cells of the normal pancreas (data not shown). In contrast, there was a moderate to strong CK 20 mRNA *in situ* hybridization signal in many cancer cells in four of five of the pancreatic cancer samples. An example of a positive cancer sample is shown in Fig. 4*D*. A CK 20 sense probe did not reveal any specific signal (Fig. 4*E*). Immunohistochemical analysis of pancreatic cancer

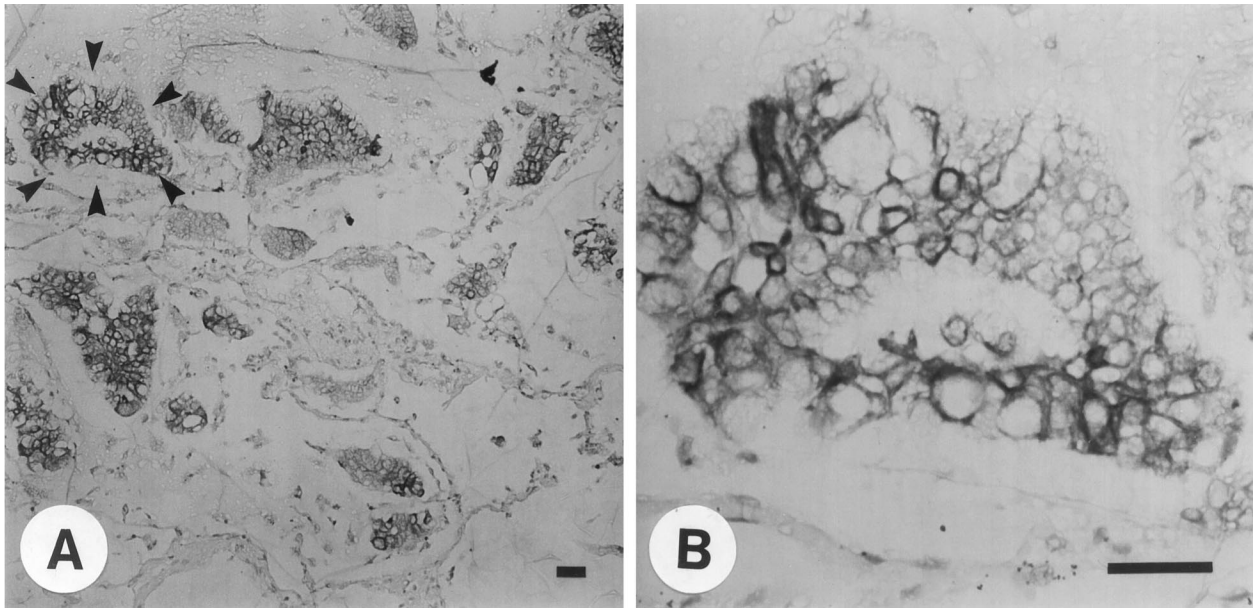

Fig. 5 CK 20 immunoreactivity in lung metastasis originating from pancreatic cancer. Strong CK 20 immunostaining is clearly evident in the pancreatic cancer cells within the lung parenchyma. *A,* low power view. *Solid arrow,* region shown under high power view in panel *B. Bar,* 25 μm.

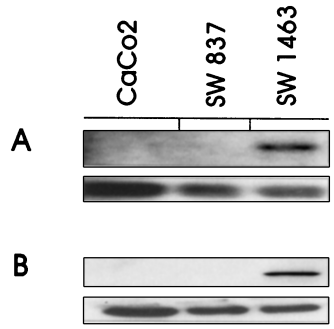

Fig. 6 Expression of CK 20 in cultured colorectal cancer cells. *A,* Northern blotting. Poly(A)$^+$-RNA (2 μg/lane) was prepared from the indicated colorectal cancer cell lines and hybridized with ^{32}P-labeled probes (500,000 cpm/ml) specific for CK 20. A 7S ribosomal cDNA probe (50,000 cpm/ml) was used as a loading and transfer control. Exposure times were 12 h for CK 20 and 4 h for 7S. *B,* Western blotting. Lysates from three colorectal cancer cell lines were probed with a highly specific CK 20 antibody (see "Materials and Methods"). ERK2 was used as a loading and transfer control. Exposure times were 2 min for CK 20 and 1 min for ERK2.

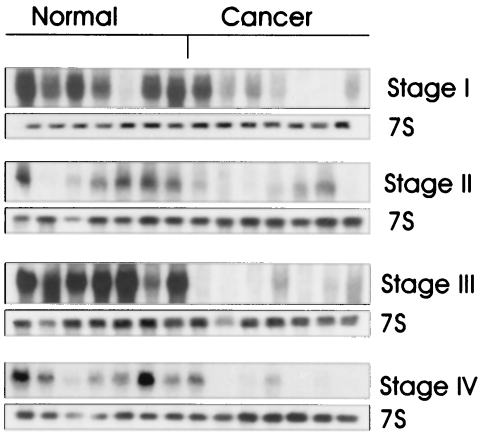

Fig. 7 Expression of CK 20 in colon tissues by Northern blotting. Total RNA (20 μg/lane) was prepared from colonic tissues from stage I through IV disease and hybridized with ^{32}P-labeled cDNA probes (500,000 cpm/ml) specific for CK 20. A 7S ribosomal cDNA probe (50,000 cpm/ml) was used as a loading and transfer control. Exposure times were 12 h for CK 20 and 4 h for 7S.

metastases was performed in two pulmonary samples, revealing strong CK 20 immunoreactivity in the metastatic cancer cells (Fig. 5).

CK 20 mRNA Expression in Colon Cancer Cell Lines and Tissues. Northern blot analysis of poly(A)$^+$ RNA revealed a strong CK 20 mRNA signal in SW 1463 cells (Fig. 6*A*). In contrast, CK 20 was below level of detection in CaCo2 and SW 837 colorectal cancer cells (Fig. 6*A*). Similarly, Western blot analysis showed a distinct band of M_r 46,000 corresponding to the size of the CK 20 protein in SW 1463, whereas SW 837 and CaCo2 did not reveal the same band (Fig. 6*B*). There was strong CK 20 immunoreactivity in SW 1463 cells (Fig. 2*D*). In contrast, CaCo2 and SW 837 did not show a CK 20 staining (data not shown).

Northern blot analysis of RNA isolated from the normal portion of the colon revealed moderate to high levels of CK 20 mRNA in 25 of 28 samples (Fig. 7). In contrast, in the corresponding cancer samples, CK 20 mRNA levels were weak to moderate in 13 of 28 samples (Fig. 7). Only 1 cancer sample in stage I disease and one cancer sample in stage IV disease exhibited high levels of CK 20 mRNA. Thus, densitometric

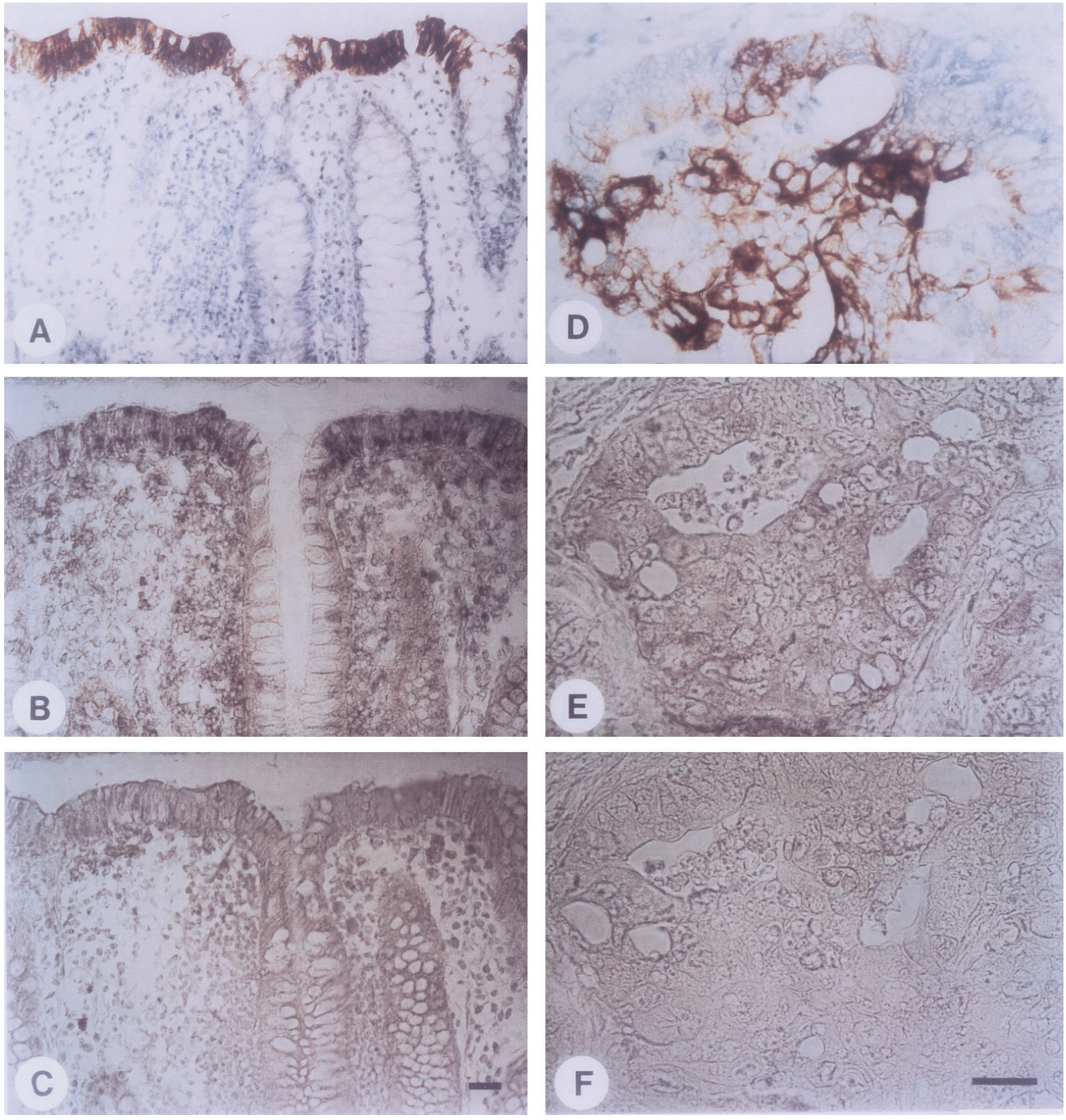

Fig. 8 Immunohistochemical analysis and *in situ* hybridization in colon tissues. Moderate to strong CK 20 immunoreactivity was evident in the apical portion of the normal colonic mucosa (*A*). Analysis of serial sections revealed that the cells that exhibited CK 20 immunoreactivity also exhibited a moderate to strong CK 20 *in situ* hybridization signal (*B*). There was strong CK 20 immunoreactivity in the cancer cells within the tumor mass (*D*). However, analysis of serial sections indicated that these cancer cells exhibited only low levels of CK 20 mRNA by *in situ* hybridization (*E*). *In situ* hybridization with the CK 20 sense probe did not reveal any specific signal (*C* and *F*). *Bar*, 50 μm.

analysis revealed that CK 20 mRNA levels were 5-fold ($P = 0.009$) higher in normal samples from stage I disease and 3.3-fold ($P = 0.009$) higher in normal samples from stage IV disease by comparison to the corresponding cancer samples. There was no significant difference in CK 20 expression between the four disease stages.

Immunohistochemical analysis revealed moderate to strong CK 20 immunoreactivity in the apical portion of the normal colonic mucosa (Fig. 8*A*). The corresponding cancer samples showed a strong CK 20 signal in the cancer cells within the tumor mass (Fig. 8*D*). By *in situ* hybridization, four of six normal samples showed a moderate signal for CK 20 mRNA

that was present in the surface epithelium and the mucosal crypts (Fig. 8*B*). In contrast, two of six colon cancer samples exhibited a weak CK 20 *in situ* hybridization signal in the cancer cells (Fig. 8*E*), although most of these samples exhibited moderately intense CK 20 immunoreactivity. This discrepancy between the *in* situ hybridization signal and the intensity of the immunostaining raises the possibility that the CK 20 protein has a long half-life. A CK 20 sense probe did not reveal any specific signal (Fig. 8, *C* and *F*).

DISCUSSION

Since its detection in 1992 (11) and the subsequent report of the complete nucleotide sequence 1 year later (17), CK 20 has become an important tool for the characterization of primary and metastatic human adenocarcinomas. It has been shown that CK 20 has a restricted range of expression in human tissues such as intestinal epithelium, gastric foveolar epithelium, urothelium, and Merkel cells in the skin. Furthermore, CK 20 has no immunological cross-reactivity with other members of the CK family, therefore allowing for a highly specific analysis of tissues by immunohistochemistry.

A large number of studies designed to detect disseminated tumor cells by using RT-PCR techniques based on the expression of CK 20 mRNA and other members of this family (34, 36–40). These reports showed a clear correlation between tumor cell spread in bone marrow, venous blood, and lymph nodes, respectively, and stage of the disease in colorectal cancer. Furthermore, Weitz *et al.* (38) reported a dissemination of tumor cells in venous blood from patients undergoing surgery for colorectal cancer disease. RT-PCR analysis using CK 20 as a marker has been especially successful in colorectal cancer (36), whereas in gastric and pancreatic cancer the findings were far less convincing (37).

In the present study, we characterized CK 20 expression in pancreatic and colonic tissues. In the normal pancreas, CK 20 immunoreactivity was faintly evident in the ductal cells. These findings contrast with previous immunohistochemical studies, which failed to demonstrate CK 20 immunoreactivity in normal pancreatic tissues (17, 18). However, in agreement with our results, CK 20 immunoreactivity has been detected in the ductal cells of the rat pancreas (18–21). Furthermore, in the present study, the expression of CK 20 mRNA in the normal pancreas was confirmed by Northern blotting. In contrast to the relatively low expression of CK 20 in the normal pancreas, the normal colon tissue samples exhibited a strong CK 20 immunoreactivity, which was restricted to the upper layer of the colonic mucosa and the mucosal crypts. These results were consistent with previous immunohistochemical studies (17, 33). In addition, in the present study, *in situ* hybridization confirmed that CK 20 mRNA was expressed in the same cells in the normal colon mucosa as shown by immunohistochemistry.

In pancreatic cancer, CK 20 immunoreactivity was extremely strong in the duct-like cancer cells. This is in contrast to previous immunohistochemical studies, which reported that only a small number of pancreatic cancers exhibited a significant level of CK 20 immunoreactivity (11, 17, 33). Our immunohistochemical findings in pancreatic cancer are supported by two observations: (*a*) CK 20 mRNA levels by Northern blot analysis were increased in a subgroup of pancreatic cancer tissues by comparison to the normal pancreas; (*b*) *in situ* hybridization analysis revealed increased CK 20 mRNA signals in the cancer cells within the pancreatic tumor mass. However, CP-like lesions adjacent to the cancer also revealed an increased expression of CK 20, indicating that elevated CK 20 expression was not specific to the cancer cells in this malignancy.

There was a good correlation between CK 20 expression by Northern blot analysis and immunoblotting in the colorectal cancer cell lines. However, in contrast to the findings in pancreatic cancer, in the colorectal cancer samples, CK 20 mRNA was decreased by comparison with the normal colon samples. This decrease was observed in all four stages and was confirmed by the relatively low CK 20 mRNA *in situ* hybridization signal in the cancer cells within the tumor mass. In contrast, normal samples exhibited markedly increased levels of CK 20 in immunohistochemistry and *in situ* hybridization in the apical mucosa. The relative abundance of CK 20 in the normal gastrointestinal mucosa by immunohistochemistry and *in situ* hybridization and its relative low abundance in the colorectal cancer cells are consistent with the observation that CK 20 expression is increased with differentiation. Nonetheless, the colon cancer cells are capable of expressing CK 20, which is the reason why CK 20 has been a useful marker for colorectal cancer (11, 17, 22–24, 31–33).

Metastatic cancer of unknown origin occasionally presents significant clinical dilemmas with respect to diagnosis and therapy. The ability of metastatic cancer cells to retain the capacity to express markers of differentiation such as CK 20 has been helpful in establishing clinically the primary source of metastatic lesions. The present study documents that CK 20 is overexpressed in a significant number of pancreatic cancers. Furthermore, we show that metastatic lesions from pancreatic cancer exhibit strong CK 20 immunoreactivity. To our knowledge, this is the first report of CK 20 overexpression in any human malignancy. We propose, therefore, that CK 20 may also be a more valuable marker for metastatic pancreatic cancer than has been appreciated previously.

REFERENCES

1. Eichner, R., Bonitz, P., and Sun, T. T. Classification of epidermal keratins according to their immunoreactivity, isoelectric point, and mode of expression. J. Cell. Biol., *98:* 1388–1396, 1984.

2. Hatzfeld, M., and Weber, K. The coiled coil of *in vitro* assembled keratin filaments is a heterodimer of type I and II keratins: use of site-specific mutagenesis and recombinant protein expression. J. Cell. Biol., *110:* 1199–1210, 1990.

3. Steinert, P. M. The two-chain coiled-coil molecule of native epidermal keratin intermediate filaments is a type I-type II heterodimer. J. Biol. Chem., *265:* 8766–8774, 1990.

4. Moll, R., Franke, W. W., Schiller, D., Geiger, B., and Krepler, R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell, *31:* 11–24, 1982.

5. Moll, R., Schiller, D. L., and Franke, W. W. Identification of protein IT of the intestinal cytoskeleton as a novel type I cytokeratin with unusual properties and expression patterns. J. Cell. Biol., *111:* 567–580, 1990.

6. Van Muijen, G. N. P., Ruiter, D. J., Franke, W. W., Achtstaetter, T., Haasnoot, W. H. B., Ponec, M., and Warnaar, S. O. Cell type heterogeneity of cytokeratin expression in complex epithelia and carcinomas

as demonstrated by monoclonal antibodies specific for cytokeratins nos. 4 and 13. Exp. Cell. Res., *162:* 97–113, 1986.

7. Wu, Y. J., Parker, L. M., Binder, N. E., Beckett, M. A., Sinard, J. H., Griffiths, C. T., and Rheinwald, J. G. The mesothelial keratins: a new family of cytoskeletal proteins identified in cultured mesothelial cells and nonkeratinizing epithelia. Cell, *31:* 693–703, 1982.

8. Osborn, M., and Weber, K. Tumor diagnosis by intermediate filament typing: a novel tool for surgical pathology. Lab. Investig., *48:* 372–394, 1983.

9. Nagle, R. B. Intermediate filaments: a review of the basic biology. Am. J. Surg. Pathol., *12* (Suppl. 1)*:* 4–16, 1988 .

10. Nagle, R. B. Intermediate filaments. Efficacy in surgical pathologic diagnosis. Am. J. Clin. Pathol., *91*(Suppl. 1)*:* 14–18, 1989 .

11. Moll, R., Loewe, A., Laufer, J., and Franke, W. W. Cytokeratin 20 in human carcinomas. Am. J. Pathol., *140:* 427–447, 1992.

12. Fuchs, E., and Green, H. Changes in keratin gene expression during terminal differentiation of the keratinocytes. Cell, *19:* 1033–1042, 1980.

13. Franke, W. W., Schiller, D. L., Moll, R., Winter, S., Schmid, E., Engelbrecht, I., Denk, H., Krepler, R., and Plazter, B. Diversity of cytokeratins. Differentiation specific expression of cytokeratin polypeptides in epithelial cells and tissues. J. Mol. Biol., *153:* 933–959, 1981.

14. Tseng, S. C. G., Jarvinen, M. J., Nelson, W. G., Huang, J. W., Woodcock-Mitchell, J., and Sun, T. T. Correlation of specific keratins with different types of epithelial differentiation: monoclonal antibody studies. Cell, *30:* 361–372, 1982.

15. Fuchs, E., Tyner, A. L., Giudice, G. J., Marchuk, D., Ray-Chaudhury, H., and Rosenberg, M. The human keratin genes and their differential expression. Curr. Top. Dev. Biol., *22:* 5–34, 1987.

16. Cooper, D., Schermer, A., and Sun, T. T. Classification of human epithelia and their neoplasma using monoclonal antibodies to keratins: strategies, applications and limitations. Lab. Investig., *52:* 243–256, 1985.

17. Moll, R., Zimbelmann, R., Goldschmidt, M. D., Keith, M., Laufer, J., Kasper, M., Koch, P. J., and Franke, W. W. The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas. Differentiation (Camb.), *53:* 75–93, 1993.

18. Bouwens, L., Lu, W. G., De Krijger, R., and Pipeleers, D. Keratins as markers of duct-to-islet cytodifferentiation in fetal pancreas. Diabetologia, *39:* A63, 1996.

19. Bouwens, L., Braet, F., and Heimberg, H. Identification of rat pancreatic duct cells by their expression of cytokeratins 7, 19 and 20 *in vivo* and after isolation and culture. J. Histochem. Cytochem., *43:* 245–253, 1995.

20. Faa, G., Van Eyken, P., Roskams, T., Miyazaki, H., Serreli, S., Ambu, R., and Desmet, V. J. Expression of cytokeratin 20 in developing rat liver and in experimental models of ductular and oval cell proliferation. J. Hepatol., *29:* 628–633, 1998.

21. Bouwens, L. Cytokeratins and cell differentiation in the pancreas. J. Pathol., *184:* 234–239, 1998.

22. Tot, T. Adenocarcinomas metastatic to the liver: the value of cytokeratins 20 and 7 in the search for unknown primary tumors. Cancer (Phila.), *85:* 171–177, 1999.

23. Lagendijk, J. H., Mullink, H., Van Diest, H., Meijer, G., and Meijer, C. J. L. M. Tracing the origin of adenocarcinomas with unknown primary using immunohistochemistry. Hum. Pathol., *29:* 491–497, 1998.

24. Maeda, T., Kajiyama, K., Adachi, E., Takenaka, K, Sugimachi, K., and Tsuneyoshi, M. The expression of cytokeratins 7, 19, and 20 in primary and metastatic carcinomas of the liver. Mod. Pathol., *9:* 901–909, 1996.

25. Berezowski, K., Stastny, J. F., and Kornstein, M. J. Cytokeratins 7 and 20 and carcinoembryonic antigen in ovarian and colonic carcinoma. Mod. Pathol., *9:* 426–429, 1996.

26. Sack, M. J., and Roberts, S. A. Cytokeratins 20 and 7 in the differential diagnosis of metastatic carcinoma in cytologic specimens. Diagn. Cytopathol., *16:* 132–136, 1997.

27. Loy, T. S., and Calaluce, R. D. Utility of cytokeratin immunostaining in separating pulmonary adenocarcinomas from colonic adenocarcinomas. Am. J. Clin. Pathol., *102:* 764–767, 1994.

28. Young, R. H., and Hart, W. R. Metastatic intestinal carcinomas simulating primary ovarian clear cell carcinoma and secretory endometrioid carcinoma: a clinicopathologic and immunohistochemical study of five cases. Am. J. Surg. Pathol., *22:* 805–815, 1998.

29. Longatto Filho, A., Bisi, H., Alves, V. A., Kanamura, C. T., Oyafuso, M. S., Bortolan, J., and Lombardo, V. Adenocarcinoma in females detected in serous effusions. Cytomorphologic aspects and immunocytochemical reactivity to cytokeratins 7 and 20. Acta Cytol., *41:* 961–971, 1997.

30. Ronnett, B. M., Kurman, R. J., Shmookler, B. M., Sugarbaker, P. H., and Young, R. H. The morphologic spectrum of ovarian metastases of appendiceal adenocarcinomas: a clinicopathologic and immunohistochemical analysis of tumors often misinterpreted as primary ovarian tumors or metastatic tumors from other gastrointestinal sites. Am. J. Surg. Pathol., *21:* 1144–1155, 1997.

31. Loy, T. S., Calaluce, R. D., and Keeney, G. L. Cytokeratin immunostaining in differentiating primary ovarian carcinoma from metastatic colonic adenocarcinoma. Mod. Pathol., *9:* 1040–1044, 1996.

32. Litle, V. R., Warren, R. S., Moore, D., II, and Pallavicini, M. G. Molecular cytogenetic analysis of cytokeratin 20-labeled cells in primary tumors and bone marrow aspirates from colorectal carcinoma patients. Cancer (Phila.), *79:* 1664–1670, 1997.

33. Miettinen, M. Keratin 20: immunohistochemical marker for gastrointestinal, urothelial, and Merkel cell carcinoma. Mod. Pathol., *8:* 384–388, 1995.

34. Wyld, D. K., Selby, P., Perren, T. J., Jonas, S. K., Allen-Mersh, T. G., Wheeldon, J., and Burchill, S. A. Detection of colorectal cancer in peripheral blood by reverse-transcriptase polymerase chain reaction for cytokeratin 20. Int. J. Cancer, *79:* 288–293, 1998.

35. Bostick, P. J., Chatterjee, S., Chi, D. D., Huynh, K. T., Giuliano, A. E., Cote, R., and Hoon, D. S. Limitations of specific reverse-transcriptase polymerase chain reaction markers in the detection of metastases in the lymph nodes and blood of breast cancer patients. J. Clin. Oncol., *16:* 2632–2640, 1998.

36. Soeth, E., Roeder, C., Juhl, H., Krueger, U., Kremer, B., and Kalthoff, H. The detection of disseminated tumor cells in bone marrow from colorectal-cancer patients by a cytokeratin-20-specific nested reverse-transcriptase-polymerase-chain reaction is related to the stage of disease. Int. J. Cancer, *69:* 278–282, 1996.

37. Soeth, E., Vogel, I., Roeder, C., Juhl, H., Marxsen, J., Krueger, U., Henne-Bruns, D., Kremer, B., and Kalthoff, H. Comparative analysis of bone marrow and venous blood isolates from gastrointestinal cancer patients for the detection of disseminated tumor cells using reverse transcription PCR. Cancer Res., *57:* 3106–3110, 1997.

38. Weitz, J., Kienle, P., Lacroix, J., Willeke, F., Benner, A., Lehnert, T., Herfarth, C., and Von Knebel Doeberitz, M. Dissemination of tumor cells in patients undergoing surgery for colorectal cancer. Clin. Cancer Res., *4:* 343–348, 1998.

39. Funaki, N. O., Tanaka, J., Ohshio, G., Onodera, H., Maetani, S., and Imamura, M. Cytokeratin 20 mRNA in peripheral venous blood of colorectal carcinoma patients. Br. J. Cancer, *77:* 1327–1332, 1998.

40. Futamura, M., Takagi, Y., Koumura, H., Kida, H., Tanemura, H., Shimokawa, K., and Saji, S. Spread of colorectal cancer micrometastases in regional lymph nodes by reverse transcriptase-polymerase chain reactions for carcinoembryonic antigen and cytokeratin 20. J. Surg. Oncol., *68:* 34–40, 1998.

41. Parker, S. L., Tong, T., Bolden, S., and Wingo, P. A. Cancer statistics. CA Cancer J. Clin., *47:* 5–27, 1997.

42. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.

43. Korc, M., Chandrasekar, B., Yamanaka, Y., Friess, H., Buechler, M. W., and Beger, H. G. Overexpression of the epidermal growth factor in human pancreatic cancer is associated with concomitant increase in the levels of epidermal growth factor and transforming growth factor α. J. Clin. Investig., *90:* 1352–1360, 1992