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Dysregulation of Carcinoembryonic Antigen Group Members CGM2, CD66a (Biliary Glycoprotein), and Nonspecific Cross-Reacting Antigen in Colorectal Carcinomas

Comparative Analysis by Northern Blot and in Situ *Hybridization*

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Genes coding for CD66a (biliary glycoprotein), carcinoembryonic antigen (CEA) group member 2 (CGM2), and nonspecific cross-reacting antigen (NCA) are members of the human CEA gene subgroup. We investigated a series of 11 colorectal carcinomas by Northern blot and isotopic *in situ* hybridization (ISH), demonstrating underexpression of CD66a and CGM2 in the majority of the carcinomas as compared with the normal mucosa, whereas NCA was overexpressed. ISH for CD66a and CGM2 mRNA revealed that large areas of the carcinomas remained without or with only faint hybridization signals. However, in every carcinoma, at least some positive foci were observed, indicating remaining cell populations that actively transcribe CD66a and CGM2. In contrast, ISH for NCA displayed strong and extensive autoradiographic signals. By analysis of step sections, foci of CD66a and CGM2 expression were shown to co-localize. Furthermore, these foci contained relatively few nuclei immunohistochemically positive for the proliferation-associated nuclear antigen Ki-67. Our data indicate a dysregulation of the three genes possibly with a common transcriptional control for CD66a and CGM2 and a different control for NCA. The focal expression of CD66a and CGM2 could be interpreted as due to a focal, incomplete, and abortive differentiation or, alternatively, as a consequence of genetic heterogeneity with foci of slow-proliferating subclones. *(Am J Pathol 1997, 151:521–530)*

The human carcinoembryonic antigen (CEA) gene subgroup contains 11 genes, among which are those coding for CD66a (biliary glycoprotein (BGP)), nonspecific cross-reacting antigen (NCA), and the product of the CEA gene family member 2 (CGM2).[1] At the genomic level, members of the CEA subgroup are arranged in a tight cluster on the long arm of chromosome 19.[2] Although several mRNA isoforms are generated in the normal colonic epithelium from the CD66a gene by alternative splicing, only one mRNA is transcribed from the CGM2 and the NCA genes, respectively.[1,3] Gene products of NCA and CGM2 as well as of other members of the CEA gene subgroup are tethered to the cytoplasmic membrane by a glycosyl-phosphatidylinositol anchor. In contrast, CD66a has a transmembrane domain followed by either a short or a long cytoplasmic tail.[4–6] Functionally, *in vitro* data indicate that CD66a is involved in signal transduction pathways.[7–9] Furthermore, it was proposed that CD66a might participate in the generation and maintenance of microvilli.[10–12] Supporting an adhesive function, CD66a and NCA have been shown *in vitro* to mediate cell aggregation via homophilic and heterophilic binding (reviewed in Ref. 6).

In previous studies, we could demonstrate by Northern blot analysis of a series of colorectal carcinomas that, as compared with the normal adjacent mucosa, CD66a and CGM2 were underexpressed in more than 80% of the cases. In contrast, in these tumors, NCA mRNA was detected in abundance, indicating a strong overexpression.[13,14] At present, the mechanisms for these phenomena are completely unknown. As mutations in the CD66a, the CGM2, and the NCA genes have not been described so far and the frequency of gross genomic alterations of chromosome 19 in colorectal cancer[15] is clearly below

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the frequencies of deregulations observed in our studies, we hypothesized that underexpression of CD66a and CGM2 and overexpression of NCA could occur through an altered transcriptional control operative in colorectal carcinomas.

Cellular changes in colorectal carcinomas have been analyzed in great detail. In the normal colonic crypt, cells spawned from the cell divisions in the basal replicative zone of the colonic crypt are pushed upwards, maturing on their way. With maturation, the cell cycle control machinery is dismantled and the crypt epithelium enters the G_0 phase of the cell cycle, which can be demonstrated immunohistochemically by negativity for the proliferation-associated nuclear antigen Ki-67.[16] Previously, we demonstrated that transcription of CD66a proceeds in an orderly fashion starting just above the replicative zone and attains a maximum in the mature superficial absorptive epithelium.[17] In contrast to the normal proliferation pattern, the growth of adenomas and carcinomas results from an expansion of the replicative zone of the normal generative basal epithelium to the upper regions of the colonic crypt.[18,19] In distinction from adenomas, carcinomas possess the additional capacity of stromal invasion, which is characterized by profound matrix remodeling. However, apart from the nuclear atypia, adenoma cells and cells of well or moderately well differentiated carcinomas share ultrastructural cytoplasmic features and the morphology of the cytoplasmic membrane with the normal immature crypt epithelium.[20–23]

As studies on the expression of CD66a and CGM2 in colorectal carcinomas were done exclusively by Northern blot analysis, to date, it remains unknown which cells within colorectal carcinomas transcribe these genes and what characterizes these cells. To answer these questions, we applied isotopic *in situ* hybridization (ISH) to a series of 11 well to moderately well differentiated colorectal carcinomas of the standard type and investigated the cellular distribution of CD66a, CGM2, and NCA expression at the transcriptional level. Specifically, we addressed the question of whether or not the deregulation of these genes could be assigned to the same cells.

Materials and Methods

Tissue Samples

Tissue from 11 well or moderately well differentiated colorectal carcinomas and from the adjacent normal mucosa were obtained from surgical resection specimens. For Northern blots, a small tissue sample was excised immediately after surgical removal and snap-frozen in liquid nitrogen. The remaining specimen was subsequently immersed in a large volume of buffered formalin (10% v/v), fixed for 12 to 24 hours at ambient temperatures, and embedded in paraffin according to standard procedures. Paraffin blocks for ISH comprised the carcinoma at the central infiltrative border and its margin as well as the adjacent normal mucosa.

RNA Extraction and Northern Blot Analysis

RNA was isolated from tissues pulverized in liquid nitrogen using Trizol reagent (Gibco/BRL, Eggenstein, Germany) according to the manufacturer's instructions. RNA was further purified by lithium chloride extraction. Nucleic acid preparations were quantified spectrophotometrically at 260 nm, and the integrity of the RNA was tested by gel electrophoresis. Fifteen micrograms of total RNA of each sample was analyzed by gel electrophoresis under denaturing conditions.[24] After ethidium bromide staining, capillary transfer to Genescreen Plus membranes was performed according to the manufacturer's instructions (NEN DuPont, Bad Homburg, Germany). For the detection of CD66a mRNA, a 396-bp *Pst*I restriction fragment of the 3′-untranslated region (3′-UTR) was used. The probe was ^{32}P labeled to a specific activity of 4×10^8 to 9×10^8 cpm/μg DNA by random priming using the Megaprime labeling system (Amersham, Braunschweig, Germany). Hybridization was carried out for 16 hours at 42°C in rapid hybridization buffer (Amersham). After hybridization, membranes were washed at 37°C twice in 2X standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) for 10 minutes, followed by two washes at 50°C in 0.1X SSC, 0.1% SDS for 15 minutes each.

Oligonucleotide probes specific to amino-terminal domains were employed for the detection of CGM2 (5′-ACTATCGAATTATAGGATATGTAAA-3′) and NCA (5′-ACAGTCTAATTGTAGGATATGTAAT-3′) transcripts. For cytokeratin 18, an oligonucleotide probe (5′-CCAATGACACCAAAGTTCTGAGGCAT-3′) specific to the 3′ end of the gene was used.[25] Oligonucleotides were ^{32}P end labeled to high specific radioactivities.[24] Hybridization was carried out in rapid hybridization buffer (Amersham) at 42°C for 12 to 16 hours. Subsequently, membranes were washed at 37°C in 2X SSC for 10 minutes followed by two washes in 1X SSC, 0.1% SDS for 15 minutes each at 52°C for CGM2, at 53°C for NCA, and at 60°C for cytokeratin 18. Filters were exposed to x-ray films at −80°C for 24 to 48 hours. For rehybridizations, membranes were stripped in 0.66 mol/L formaldehyde, 0.05 mol/L Tris/HCl (pH 7.9), 0.1% SDS for 15 minutes at room temperature. Signals were quantified by scanning of x-ray films using an AGFA Arcus scanner and subsequently analyzed using the densitometry software IMAGE 1.42 (public domain software of the National Technical Information Service). Expression levels of CD66a, CGM2, NCA, and cytokeratin 18 were calculated from the integer over the mRNA signals as previously described.[13,14] Tumor to normal ratios were assessed using the formula $RE_y = Y_{tu} \times Y_N^{-1} \times Cyt_N \times Cyt_{TU}^{-1}$, where Y is the expression of either CD66a, CGM2, or NCA and *Cyt* is the expression of cytokeratin 18 in tumor (TU) and normal tissue (N), respectively.

Generation of Riboprobes for in Situ Hybridization

For CD66a and NCA, the corresponding 3′-UTR of the human cDNAs subcloned into the Bluescript II KS+ vec-

tor (Stratagene, Heidelberg, Germany) were employed.[4,13] The full-length human CGM2 cDNA (kind gift of Dr. J. Thompson, Institute of Immunobiology, University of Freiburg, Freiburg, Germany) was subcloned into the *Eco*RI and *Hind*III restriction sites of the Bluescript II KS+ vector. Riboprobes were then generated by run-off transcription from linearized plasmid with [^{35}S]UTP using T3 or T7 RNA polymerase (Boehringer Mannheim, Mannheim, Germany), followed by alkaline hydrolysis to allow better tissue penetration. On average, riboprobes were labeled to specific radioactivities of 4×10^7 to 6×10^7 cpm/μg RNA. The specificity of these riboprobes had previously been confirmed by Northern blot.[13,14]

In Situ *Hybridization and Autoradiography*

Four-micron step sections were mounted on 3-aminopropyltriethoxysilane (APES)-coated slides, deparaffinized in xylene for 12 hours, and rehydrated to phosphate-buffered saline (PBS) through graded ethanol solutions. Pretreatment with 0.2 mol/L HCl for 20 minutes was followed by digestion with Pronase (Boehringer Mannheim) at 600 μg/ml for 10 minutes at room temperature, postfixation with freshly prepared paraformaldehyde (4% w/w) in PBS for 20 minutes on ice, and acetylation. Rinsing in PBS followed each step. After dehydration in graded ethanol solutions and drying, hybridization was performed at 52°C for 16 to 18 hours with 4×10^5 cpm antisense or sense probe in 50 μl of hybridization solution containing 50% formamide, 10% dextran, 10 mmol/L Tris/HCl (pH 7.5), 10 mmol/L Na_3PO_4 (pH 6.8), 300 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L dithiothreitol, 1 mg/ml yeast transfer RNA (Boehringer Mannheim) and 1X Denhardt's solution (Sigma, Deisenhofen, Germany). Slides were subsequently washed in modified hybridization buffer (without yeast tRNA and Denhardt's solution) for 4 hours at 52°C, subjected for 20 minutes at 37°C to RNAse A (20 μg/ml; Boehringer Mannheim) followed by additional washing for 30 minutes at 37°C in 100 mmol/L Tris/HCl (pH 7.5), 500 mmol/L NaCl, 1 mmol/L EDTA and two washes for 30 minutes each at room temperature in 2X SSC and 0.2x SSC. After dehydration in graded ethanol solutions/600 mmol/L ammonium acetate, drying slides were coated with Kodak NTB-2 photographic emulsions diluted at equal volumes with 600 mmol/L ammonium acetate. Slides were exposed for autoradiography at 4°C for 28 to 42 days, developed in Kodak D19 developer for 5 minutes, rinsed in 1% acetic acid, and fixed in Kodak fixer for 6 minutes. After washing in tap water, slides were stained with hematoxylin and coverslipped using a synthetic mounting medium.

Immunohistochemistry

Deparaffinized slides were rehydrated in Tris-buffered saline (50 mmol/L Tris, 150 mmol/L NaCl, pH 7.4) and blocked with normal goat serum (Dako, Glostrup, Denmark) diluted 1/20 in Tris-buffered saline. Monoclonal antibody MiB-1 (anti Ki-67; Dianova, Hamburg, Germany) was applied at 10 μg/ml for 16 hours at 4°C after microwave treatment at 500 W for 30 minutes in 10 mmol/L sodium citrate (pH 6.0). The detection was carried out with alkaline phosphatase-anti-alkaline phosphatase (APAAP) reagents and color development with new fuchsin according to standard procedures.

Microscopic Evaluation of in Situ *Hybridization and Immunohistochemistry*

For the semiquantitative analysis of ISH, the slides were viewed in representative fields at ×250 magnification using an ocular eye grid of 100 μm square length with evaluation of 120 to 220 squares for each hybridization. The autoradiographic signals overlying squares containing atypical epithelium were evaluated and by arbitrary classification were scored as strongly (>100 grains above background), moderately (30 to 100 grains), or weakly (<30 grains) positive or negative. Autoradiographic signals from hybridizations with different probes and with the immunohistochemical stain for Ki-67 were correlated at the cellular level by taking microphotographs of selected corresponding regions in 4-μm sections immediately adjacent to each other. The microphotographs were subsequently projected in parallel for direct comparison, and the distributions of autoradiographic signals for different probes were recorded.

Results

Northern Blot Analysis of Colorectal Carcinomas Demonstrates Underexpression of CD66a and CGM2 but Overexpression of NCA

Northern blot analyses were performed with total RNA prepared from 11 colorectal carcinomas and their adjacent normal mucosa. Confirming the specificity of the cDNA probe and the oligonucleotide probes, hybridizations with RNA from the normal mucosa yielded the expected transcripts for CD66a, CGM2, and NCA of 3.9, 2.5, and 3.0 kb, respectively (Figure 1).

On the rationale that carcinomas and normal mucosa contain different amounts of epithelia in relation to stroma, the filters were rehybridized with an oligonucleotide probe specific for cytokeratin 18,[25] and the expression levels of CD66a, CGM2, and NCA were quantified as ratios according to the formula given in Materials and Methods. Based on these calculations, complete loss or marked underexpression ($RE \leq 0.3$) was observed in 6 of 11 carcinomas (55%) for CD66a and in 90% of the specimens (9 of 10) for CGM2 (see Figure 5, A and B). Moderate decrease of expression ($RE > 0.3$ and < 0.8) was found in three tumors for CD66a and in one tumor for CGM2, respectively. Only one of the carcinomas showed overexpression of CD66a (T1, see Figure 5A). In contrast, marked overexpression of NCA (*RE* in the range of 1.3 to 3.6) was observed in 8 of 11 carcinomas (73%). However, only weak overexpression was found in tumor 6

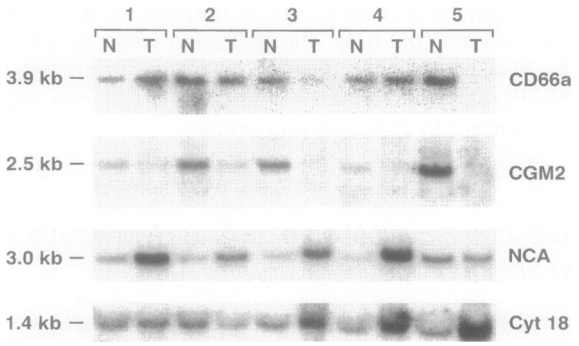

Figure 1. Northern blot analysis of five representative colorectal carcinomas (T) and their adjacent normal mucosa (N) for CD66a, CGM2, NCA, and cytokeratin 18. A 15-μg aliquot of total RNA was electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized with a ^{32}P-labeled 396-bp restriction fragment from the 3′-UTR of the CD66a cDNA or ^{32}P-labeled oligonucleotides specific for the amino-terminal domains of CGM2 and NCA, respectively, and to the 3′ end of cytokeratin 18. The results from densitometric evaluations for all carcinomas with corrections for cytokeratin 18 are represented in Figure 5.

(*RE* = 1.1), and marked underexpression of NCA (*RE* ≤ 0.3) was observed in 2 of 11 carcinomas (T5 and T9, see Figure 5C).

In Situ *Hybridization Reveals a Focal Expression of CD66a, CGM2, and NCA in the Atypical Epithelium of Carcinomas*

In a second set of experiments, we carried out ISH to resolve the summative hybridization signals from Northern blots morphologically. Riboprobes were hybridized to paraffin sections from the carcinomas and their adjacent normal mucosa. Validating the specificity of the results of the Northern blots, the autoradiographic signals were restricted to the normal colonic epithelium and the atypical epithelium of the carcinomas. Hybridizations with sense probes always remained negative.

When probes for CD66a, CGM2, and NCA mRNAs were applied to normal colonic mucosa, dense clusters of autoradiographic grains were observed overlying the absorptive surface epithelium and the apical crypt epithelium (Figure 2). This expression pattern has recently been published for CD66a,[17,26] a finding that hereby can be extended to CGM2 and NCA, respectively.

Hybridizations for CD66a and CGM2 revealed large areas of the carcinomas without or with only faint hybridization signals (Figure 3). Furthermore, autoradiographic signals for CD66a and CGM2 overlying the atypical epithelium in many carcinomas were very irregular in their

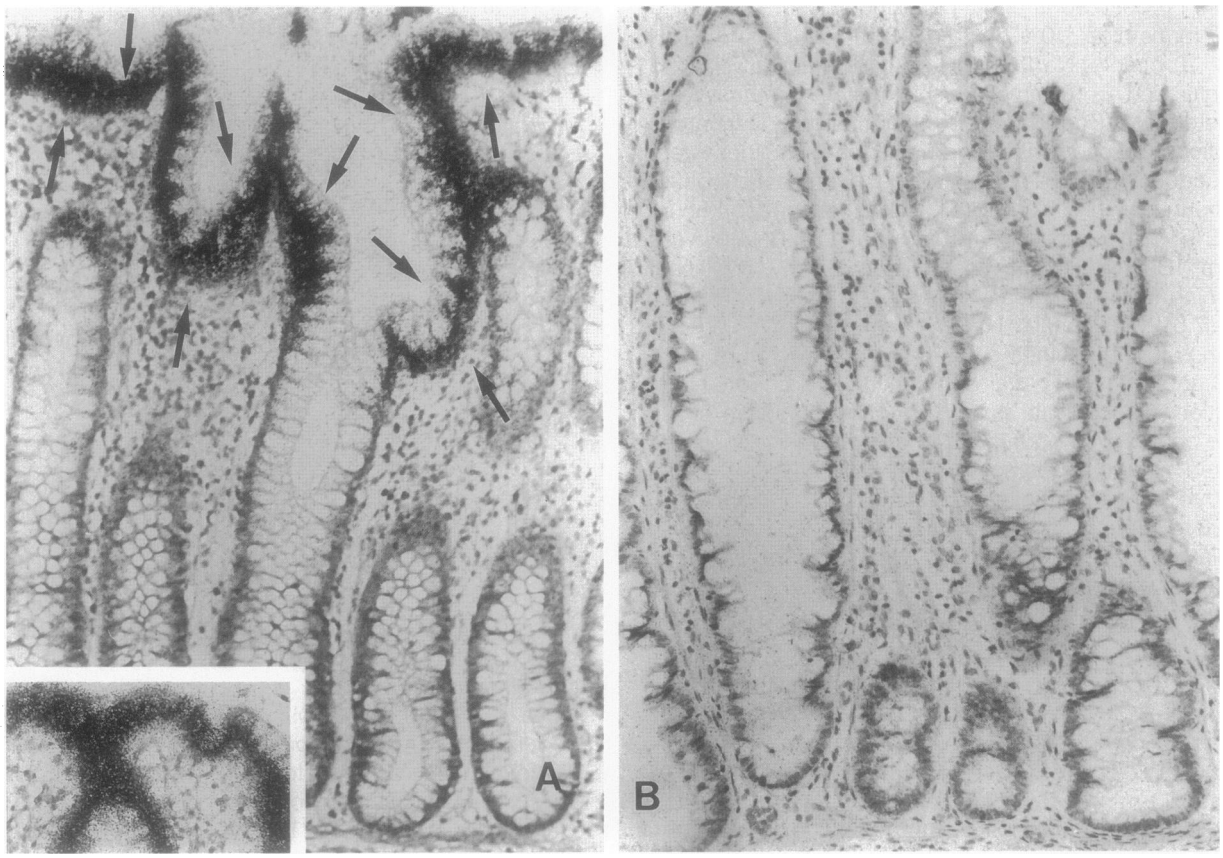

Figure 2. A: CGM2 expression in the apical crypt epithelium and the superficial absorptive epithelium of normal colonic mucosa (**arrows**) using the antisense probe. See higher magnification **inset** for details. **B:** Hybridization with the sense probe as control. Autoradiographic exposure, 42 days: magnification, ×362 and ×725 (**inset**).

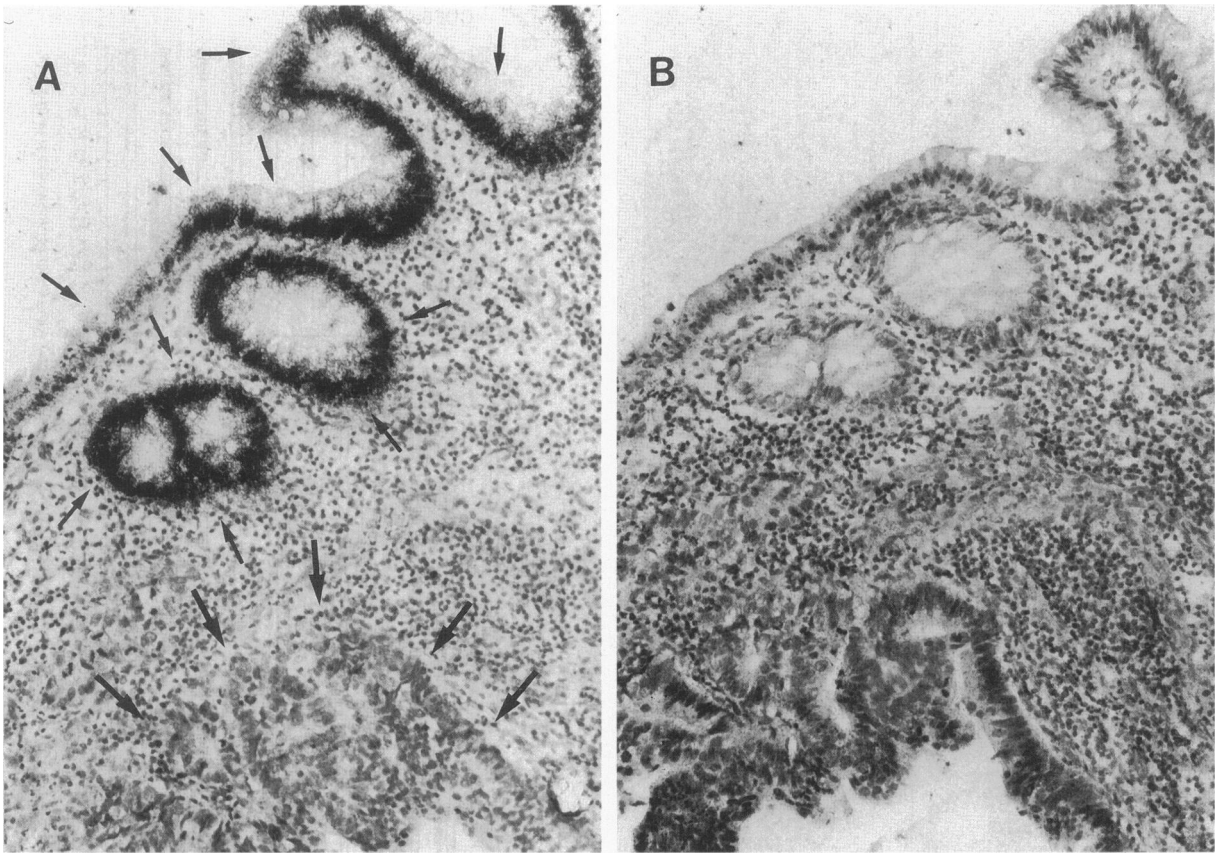

Figure 3. Underexpression of CD66a in a colorectal carcinoma. **A:** Hybridization with the antisense probe. Note the absence of autoradiographic grains over the carcinoma cells (demarcated by **thick arrows**, bottom) whereas strong autoradiographic signals are seen overlying the apical crypt epithelium and the superficial absorptive epithelium (demarcated by **thin arrows**, **top**). Less intensive signals are found over mature absorptive cells in the middle region between crypts. **B:** Same region hybridized with the sense probe as control. Autoradiographic exposure, 42 days; magnification, ×362.

distribution. However, at least some positive foci were observed in every carcinoma, indicating that, despite a net underexpression of CD66a and CGM2, there remain cell populations that actively transcribe CD66a and CGM2 (Figure 4). A careful analysis of the underlying conventional histology by the pathologists in our group gave no clue to the nature of these cell populations; ie, they were clustered neither at the infiltrative margins nor in the central regions or preferentially within lymphatic vessels. In contrast, hybridizations for NCA resulted in stronger and far more extensive autoradiographic signals over the atypical epithelium. However, some small foci with weaker hybridization signals for NCA could be demonstrated in the majority of the cases (see Figure 6A).

By semiquantitative analysis, the hybridization signals were compared with the Northern blot data. The summarized results presented in Figure 5 allow the general conclusion that carcinomas with underexpression of CD66a or CGM2 as demonstrated in Northern blots displayed fewer autoradiographically positive fields with signals frequently of lesser intensity. For NCA, overexpression in Northern blots was generally accompanied by intense positive autoradiographic signals covering most of the tissue areas. A strict correlation, however, is not tenable.

CD66a and CGM2 Are Co-Expressed in the Same Subpopulations of Carcinoma Cells and Do Not Co-Localize with Immunohistochemical Stains for the Proliferation Antigen Ki-67

In view of the focal nature of the expression of CD66a, CGM2, and NCA, we addressed the question of co-expression of these transcripts. Four cases were selected for further analyses, and 4-μm sections were strictly taken in sequence. ISH resulted in a series of slides with hybridization signals for NCA 4 μm adjacent to CD66a and 4 μm adjacent to CGM2, respectively. The slides were screened for areas with focal expressions, and from each case, three or more regions were microphotographed. These microphotographs were subsequently projected in parallel allowing the direct comparison of cell populations. Reviewing pairs of microphotographs demonstrated that the autoradiographic signals for CD66a and CGM2 consistently co-localized (compare Figure 6, C and D). Furthermore, cell populations expressing CD66a and CGM2 always showed expression of NCA. However, as would be expected from the net overexpression of NCA, autoradiographic signals for NCA were found in many regions

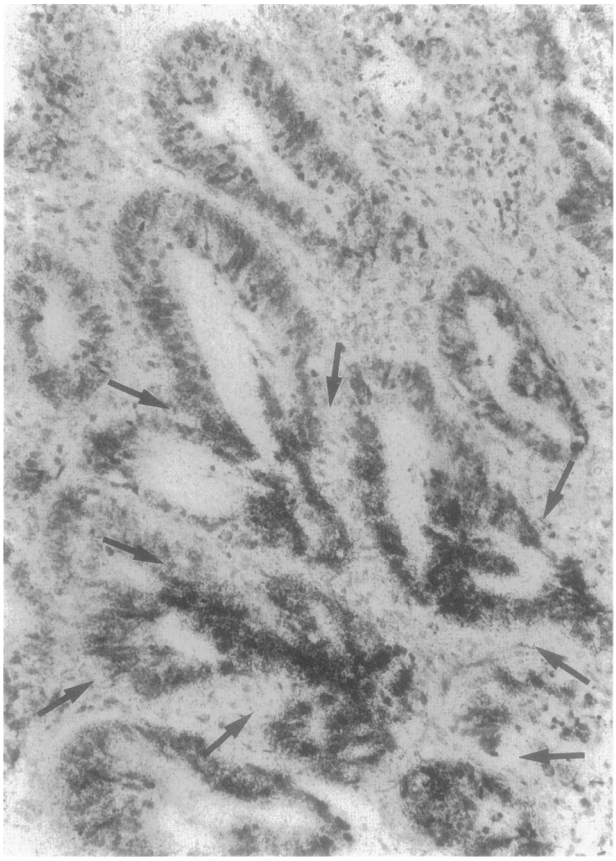

Figure 4. CD66a expression in colorectal carcinomas. Focal pattern with some areas without and some areas with strong autoradiographic signals (demarcation by **arrows**). Autoradiographic exposure, 36 days; magnification, ×362.

negative for CD66a and CGM2 (compare Figure 6, A and B).

Finally, monoclonal antibody MiB-1 specific for the Ki-67 antigen was applied to the step sections to relate the focal expression of CD66a, CGM2, and NCA to the proliferative status of cells. Areas with clearly demarcated autoradiographic clusters were sought, microphotographed, and compared in parallel projection to microphotographs of the corresponding regions stained for the proliferation antigen Ki-67. The selected foci contained relatively few stained nuclei as compared with areas with less autoradiographic labeling, indicating that carcinoma cells focally expressing CD66a and CGM2 are predominantly in a G_0 state (compare Figure 6, C, D, and E). In contrast, even highly proliferative areas of the carcinomas with a large fraction of nuclei stained for the Ki-67 antigen displayed fairly strong autoradiographic signals for NCA. However, the comparison of the nuclear (ie, the immunohistochemical staining) and the cytoplasmic features (ie, autoradiographic signals) in step sections can be problematic as the cytoplasm and the nuclei of a given cell are not necessarily retrieved in the adjacent plane. Also, our observations naturally can make use only of clearly demarcated foci of autoradiographic signals, which do not constitute the majority of regions positive for CD66a and CGM2 expression.

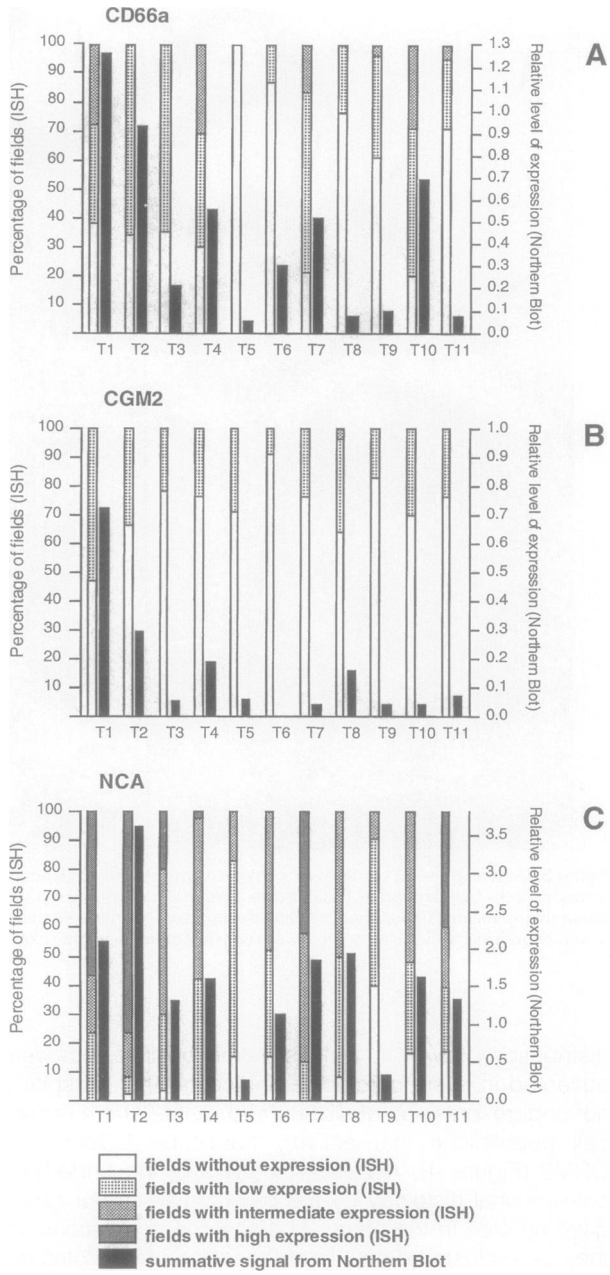

Figure 5. Comparison of semiquantitative evaluation of ISH and quantified Northern data. The percentage of fields with absent (□), low (▨), moderate (▩), or strong (■) autoradiographic labeling is given by layered columns to the left. Relative levels of CD66a (**A**), CGM2 (**B**), and NCA (**C**) are represented in columns to the right (■) determined by Northern blot analyses. Due to technical reasons, no Northern blot data were available for the relative levels of CGM2 expression in tumor 6 (T6).

Discussion

In this communication, we describe for the first time the patterns of mRNA expression of the CEA family members CD66a, CGM2, and NCA at the cellular level in colorectal carcinomas. For these transcripts, a marked dysregulation has been demonstrated as a frequent event with more than 80% of the carcinomas affected.[13,14] In this study, a series of 11 standard-type well to moderately well differentiated colorectal carcinomas and their adja-

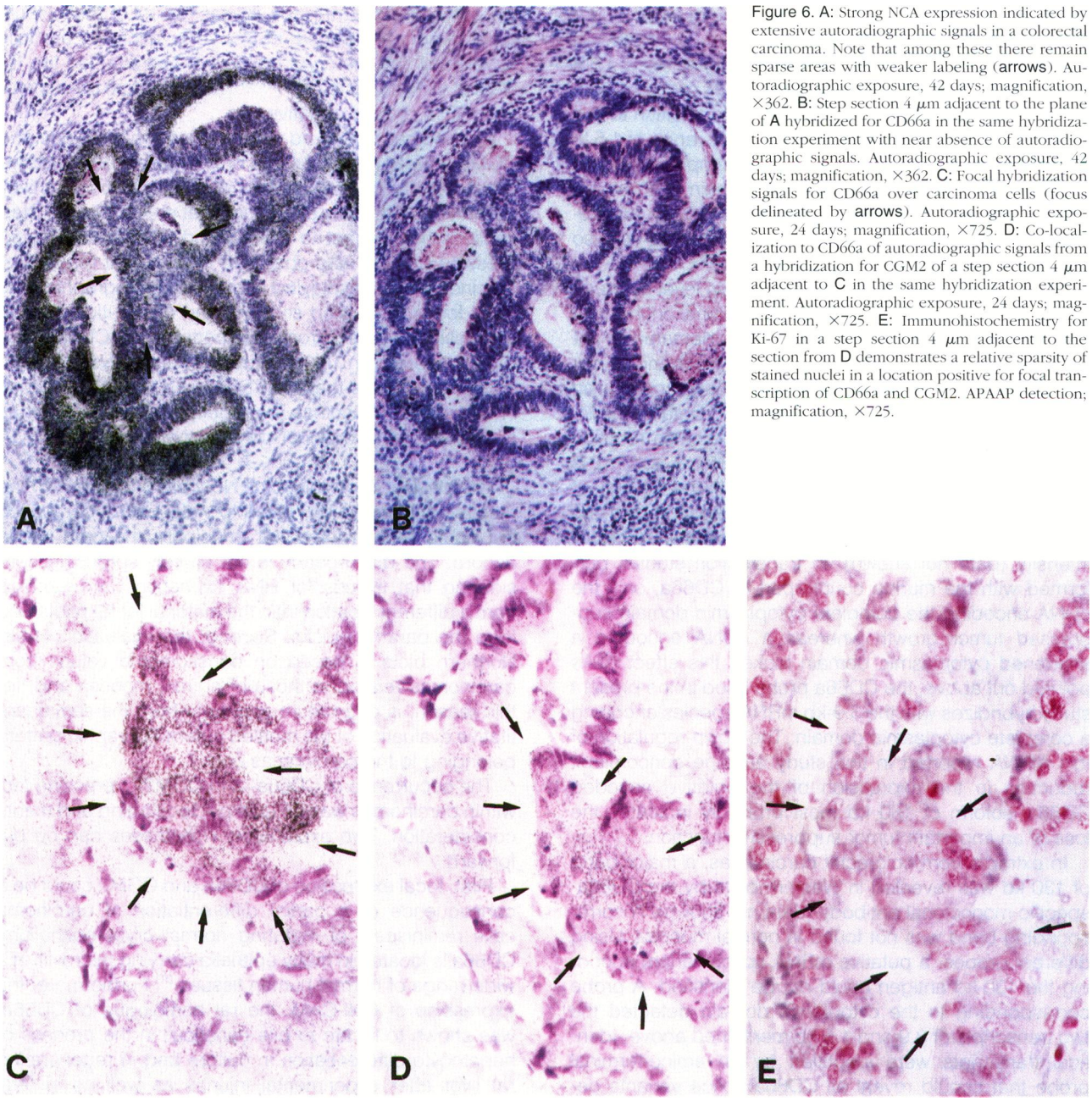

Figure 6. A: Strong NCA expression indicated by extensive autoradiographic signals in a colorectal carcinoma. Note that among these there remain sparse areas with weaker labeling (arrows). Autoradiographic exposure, 42 days; magnification, ×362. B: Step section 4 μm adjacent to the plane of A hybridized for CD66a in the same hybridization experiment with near absence of autoradiographic signals. Autoradiographic exposure, 42 days; magnification, ×362. C: Focal hybridization signals for CD66a over carcinoma cells (focus delineated by arrows). Autoradiographic exposure, 24 days; magnification, ×725. D: Co-localization to CD66a of autoradiographic signals from a hybridization for CGM2 of a step section 4 μm adjacent to C in the same hybridization experiment. Autoradiographic exposure, 24 days; magnification, ×725. E: Immunohistochemistry for Ki-67 in a step section 4 μm adjacent to the section from D demonstrates a relative sparsity of stained nuclei in a location positive for focal transcription of CD66a and CGM2. APAAP detection; magnification, ×725.

cent normal mucosae were analyzed by isotopic ISH and Northern blot analyses. Consistent with our previous reports, underexpression of CD66a and CGM2 as well as overexpression of NCA was observed by Northern blot with comparable frequencies.

When conclusions are drawn from mRNA expression patterns revealed by isotopic ISH, two aspects have to be considered. First, for some of the CEA family members, different mRNA splice variants have been described. Whereas only one mRNA species appears to exist for NCA, two splice variants have been described for CGM2. Results from Northern blots, cDNA cloning experiments, and polymerase chain reaction amplifications suggest the existence of 10 CD66a splice variants.[26] Second, the mRNA expression patterns do not always reflect the protein expression patterns. For example, there is no significant quantitative difference between the CEA mRNA levels in normal colonic mucosa *versus* colorectal carcinomas.[13] However, immunohistochemical methods reveal a significantly higher staining intensity in colorectal carcinomas as compared with normal colonic mucosa.[27] Furthermore, CEA tissue concentrations are up to 400 times higher in colorectal carcinomas in comparison with normal colonic mucosa.[28]

As only one NCA transcript has been described so far, isotopic ISH should reflect the actual mRNA levels. For CGM2, a transcript of 3.2 kb has been observed in some colorectal carcinomas in addition to the major transcript of 2.5 kb.[14] However, as isotopic ISH revealed a down-regulation of CGM2 mRNA in most carcinoma speci-

mens, the 3.2-kb transcript appears to be of minor quantitative significance. For the detection of mRNA(s) encoding CD66a, a probe complementary to the 3′-UTR was applied. In Northern blots, the 3′-UTR probe reveals a major CD66a transcript of 3.9 kb.[4,13] In addition to this transcript, a second major transcript in the range of 1.5 to 2.2 kb has been detected by probes complementary to sequences encoding either the A2 domain or the cytoplasmic domain of CD66a.[4,5,13,29] According to our previous results, the second transcript of 1.5 kb is also down-regulated in colorectal carcinomas.[13]

When CD66a is analyzed on the protein level by Western blots using a CD66a-specific monoclonal antibody, a major band corresponding to 160 kd is detected in extracts from normal colonic mucosa.[30] According to our previous results, the CD66a isoform of 160 kd contains an A2 and complete cytoplasmic domain.[31] Data of the literature and our unpublished polymerase chain reaction amplification data suggest that the 160-kd isoform is encoded by the 3.9-kb mRNA species. In the majority of colorectal carcinomas, the band corresponding to the 160-kd isoform is either absent or significantly reduced in intensity (data not shown). In transfection studies performed with the murine counterpart of CD66a, only the cDNA encoding the complete cytoplasmic domain suppressed tumor growth whereas a cDNA encoding a shortened cytoplasmic domain lacked this effect.[32] As pointed out above, the CD66a probe used in the present study hybridizes with the 3.9-kb mRNA species encoding a complete cytoplasmic domain. The down-regulation of this mRNA reported in this study and the concomitant reduction in the expression of the putative encoded CD66a isoform of 160 kd most probably indicates the loss of an important tumor suppressive function.

In extracts from colorectal carcinomas, a major band of 130 kd was revealed in Western blots by the CD66a-specific monoclonal antibody (data not shown). A corresponding band was not found in normal mucosa. Using different probes, a putative mRNA splice variant encoding the 130-kd antigen could not be identified. A probe corresponding to the cytoplamic domain detected the two transcripts of 3.9 and 1.5 kb mentioned above. Identical transcripts were identified by an amino-terminal probe that should reveal all CD66a splice variants described so far (data not shown). As mentioned above, both transcripts are down-regulated in colorectal carcinomas.

At present, the nature of the 130-kd antigen remains obscure. Possibly, the CD66a antibody cross-reacts with an epitope of a different member of the CEA family that becomes available due to incomplete glycosylation. The antigen may be identical with the 128-kd CEA variant described previously.[33] The presence of the 130-kd antigen in colorectal carcinomas may explain our immunohistochemical results, which do not reflect the mRNA expression data. In contrast to the isotopic ISH results presented here, the monoclonal CD66a antibody shows a positive staining reaction in the majority of colorectal carcinomas.

As demonstrated by ISH, the transcription of CD66a, CGM2, and NCA begins with maturation in an orderly concomitant pattern in the apical region of the colonic crypt in normal mucosa. In this zone, microvilli are fully developed and the crypt epithelium has attained the G_0 state of the cell cycle.[16,20,34–36] The immature basal crypt epithelium proved negative.

Carcinomas displayed underexpression of CD66a and CGM2 in large areas without or with only little transcriptional activity, but small foci of transcriptional activity were observed in all carcinomas that co-localized in step sections. In contrast to CD66a and CGM2, an overexpression of NCA was observed in the great majority of carcinoma cells displaying strong autoradiographic positivity for NCA. In step sections, the distribution of NCA expression differed from the patterns of CD66a and CGM2 expression. In conclusion, our analysis of step sections suggests that the CD66a and the CGM2 genes in colorectal carcinomas are regulated similarly whereas a different transcriptional control operates on the NCA gene.

The attempt to correlate the Northern blot data with the results obtained by ISH failed to yield a strict correlation but could demonstrate that principally the data are in accord. The discrepancy is not entirely surprising considering that tissues for RNA extraction were excised from a different location than the material for ISH obtained form the paraffin blocks. Second, the evaluation of the Northern blots is based on densitometric values from carcinomas related to those of normal mucosa and, for this reason, is of a nature disparate from the semiquantitative evaluation of the mottled autoradiographic pattern belonging to the carcinomas only.

The occurrence of CD66a- and CGM2-expressing foci within carcinomas was an unexpected finding and merits consideration. Two explanatory hypotheses can be put forward.

First, focal expression of CD66a and CGM2 could be a consequence of a partial differentiation of carcinoma cells reminiscent of maturing normal crypt epithelium. CD66a is located in many epithelia carrying microvilli in a wide range of normal human tissues.[30] Furthermore, the expression of Cell-CAM, the rat homologue of CD66a, was shown to locate to bile canaliculi in the process of hepatocyte differentiation in culture and in regenerating rat liver after experimental injuries as well as in fetal development and apically in cultured enterocytes, suggesting a functional role in the generation of microvilli.[10–12] Possibly, the inability of carcinoma cells to transcribe sufficient quantities of CD66a is among the causes for the inadequately and incompletely developed microvilli in these cells,[20–23] and perhaps even for the partial loss of cellular polarity. In addition, *in vitro* studies make CD66a a likely participant in signal transduction pathways[7–9] that, by the lack of CD66a in carcinoma cells, might be compromised.

An attractive unifying scenario is that CD66a links the elaboration of microvilli and cellular polarity to signal transduction, which confers a control of cellular proliferation inducing the transition to the G_0 state during the process of maturation in the normal crypt epithelium. To juxtapose similarities and dissimilarities in the light of the above hypothesis, the expression patterns of CD66a,

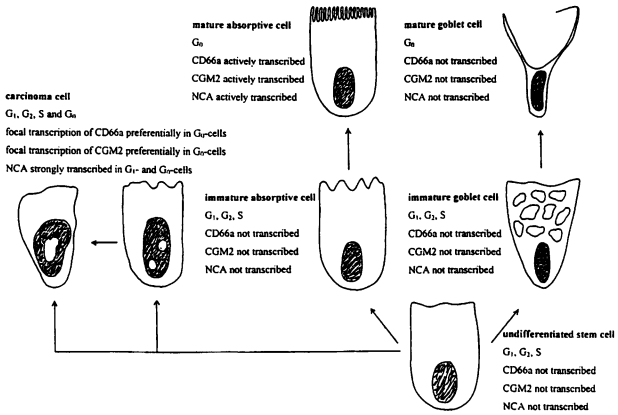

Figure 7. Schematic overview representing the ultrastructural phenotype and patterns of CD66a, CGM2, and NCA expression as derived from the hybridization experiments of normal cells in various stages of differentiation in comparison to well differentiated (left) or moderately well differentiated (far left) carcinoma cells.

CGM2, and NCA and the phenotype of immature and mature normal crypt epithelium and of well and moderately well differentiated carcinoma cells are schematically represented in Figure 7. Undifferentiated stem cells and immature absorptive cells as well as well to moderately well differentiated carcinoma cells proliferate and have underdeveloped microvilli. These cell types lack the expression of CD66a and CGM2. Presumably, in the normal crypt epithelium, maturation of stem cells and immature cells follows a programmed series of events: transcription of CD66a, CGM2, and NCA is turned on, microvilli are formed, and proliferation ceases. On the other hand, in carcinoma cells, microvilli are not fully developed,[20–23] and only a partial, abortive differentiation is attained in small foci expressing CD66a and CGM2. By analysis of step sections, these foci were shown to co-localize preferentially with the low expression of the proliferation-associated nuclear antigen Ki-67, which according to the above hypothesis could be interpreted as growth arrest induced by CD66a.

Alternatively, focal expression may reside in subclones that, by expressing CD66a and possibly CGM2, are at a disadvantage in proliferation or survival in comparison with the non-expressor clone(s).

In the course of colorectal tumorigenesis, epithelial cells acquire a sequence of genetic defects finally resulting in genetic instability and heterogeneity.[15] A particular genetic defect in a tumor cell may be associated with the down-regulation of CD66a and CGM2, respectively. As CD66a-negative carcinoma cells proliferate faster[32,37] or may escape apoptosis, expressor cells would be overgrown by the subclone(s) of non-expressor cells. In comparison with the former hypothesis, this model would account for the finding that, in carcinoma cells, the regulation of CD66a and CGM2 expression appears to be uncoupled from the regulation of NCA expression. To test this model, it will be important to establish at which point of the adenoma to carcinoma sequence the dysregulation of these CEA family members becomes apparent.

In conclusion, our data indicate a profound transcriptional dysregulation of CD66a, CGM2, and NCA in colorectal carcinomas. Conceivably, in colorectal carcinomas, the regulation for CD66a and CGM2 proceeds by the same mechanisms of transcriptional control whereas a different mechanism might operate on the NCA gene. The focal expression could occur in the context of an incomplete and abortive differentiation within carcinomas or, alternatively, result from genetic heterogeneity in which CD66a- and CGM2-negative subclones would overgrow the positive subclones.

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References

1. Hammarström S, Khan WN, Teglund S, Hammarström ML, Ramos T, Baranov W, Yeung MM, Frängsmyr L: The carcinoembryonic antigen family. Structures of Antigens, vol 2. Edited by MHV VanRegenmortel. Boca Raton, FL, CRC Press, 1993, pp 341–376
2. Brandriff BF, Gordon LA, Olsen AS, Mohrenweiser HW, Fertitta A, Carrano AV, Trask BJ: Order and genomic distances among members of carcinombyonic antigen (CEA) gene family determined by fluorescence *in situ* hybridization. Genomics 1992, 12:773–779
3. Barnett TR, Drake L, Pickle W: Human biliary glycoprotein gene: characterization of a family of novel alternatively spliced RNAs and their expressed proteins. Mol Cell Biol 1993, 13:1273–1282
4. Hinoda Y, Neumaier M, Hefta SA, Drzeniek Z, Wagener C, Shively L, Hefta LJF, Shively JE, Paxton RJ: Molecular cloning of a cDNA coding biliary glycoprotein I: primary structure of a glycoprotein immunologically crossreactive with carcinoembryonic antigen. Proc Natl Acad Sci USA 1988, 85:6959–6963
5. Barnett TR, Kretschmer A, Austen DA, Goebel SJ, Hart JT, Elting JJ, Kamarck ME: Carcinoembryonic antigens: alternative splicing accounts for the multiple mRNAs that code for novel members of the carcinoembryonic antigen family. J Cell Biol 1989, 108:267–276
6. Thompson JA, Grunert F, Zimmermann W: Carcinoembryonic gene family: molecular biology and clinical perspectives. J Clin Lab Anal 1991, 5:344–366
7. Blikstad I, Wikström T, Aurivillius M, Öbrink B: C-CAM (Cell-CAM 105) is a calmodulin binding protein. FEBS Lett 1992, 302:26–30
8. Skubitz KM, Ducker TP, Skubitz APN, Goueli SA: Antiserum to carcinoembryonic antigen recognizes a phosphotyrosine-containing protein in human colon cancer cell lines. FEBS Lett 1993, 318:200–204
9. Brümmer J, Neumaier M, Göpfert C, Wagener C: Association of $pp60^{c\text{-}src}$ with biliary glycoprotein (CD66a), an adhesion molecule of the carcinoembryonic antigen family downregulated in colorectal carcinomas. Oncogene 1995, 11:1649–1655
10. Odin P, Öbrink B: The cell-surface expression of the cell adhesion molecule Cell-CAM 105 in rat fetal tissues and regenerating liver. Exp Cell Res 1988, 179:89–103
11. Tingström A, Öbrink B: Distribution and dynamics of cell surface-associated Cell-CAM 105 in cultured rat hepatocytes. Exp Cell Res 1989, 185:132–142
12. Hansson M, Blikstad I, Öbrink B: Cell-surface location and molecular properties of Cell-CAM 105 in intestinal epithelial cells. Exp Cell Res 1989, 181:63–74
13. Neumaier M, Paululat S, Chan A, Matthaes P, Wagener C: Biliary glycoprotein, a potential human cell adhesion molecule, is down-regulated in colorectal carcinomas. Proc Natl Acad Sci USA 1993, 90:10744–10748
14. Thompson J, Zimmermann W, Nollau P, Neumaier M, Weber-Arden J, Schrewe H, Craig I, Willcocks T: CGM2, a member of the carcinoem-

bryonic antigen gene family, is down-regulated in colorectal carcinomas. J Biol Chem 1994, 269:32924–32931
15. Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 1990, 61:759–767
16. Cattoretti G, Becker MHG, Dey G, Duchrow M, Schlüter C, Galle J, Gerdes J: Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MiB1 and MiB3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 1992, 168: 357–363
17. Frängsmyr L, Baranov V, Prall F, Yeung M-W, Wagener C, Hammarström S: Cell- and region-specific expression of biliary glycoprotein and its messenger RNA in normal human colonic mucosa. Cancer Res 1995, 55:2963–2967
18. Lipkins M: Proliferation and differentiation of normal and neoplastic cells in the colon of man. Cancer 1971, 28:38–40
19. Wiebecke B, Brandts A, Eder M: Epithelial proliferation and morphogenesis of hyperplastic adenomatous and villous polyps of the human colon. Virchows Arch A Pathol Anat Histol 1974, 364:35–49
20. Kaye GI, Fenoglio CM, Pascal RR, Lane N: Comparative electron microscopic features of normal, hyperplastic, and adenomatous human colonic epithelium. Gastroenterology 1973, 64:926–945
21. Mughal S, Filipe MI: Ultrastructural study of the normal mucosa and the adenoma-cancer sequence in the development of familial polyposis coli syndrome. J Natl Cancer Inst 1978, 60:753–768
22. Hickey WF, Seiler MW: Ultrastructural markers of colonic adenocarcinomas. Cancer 1981, 47:140–145
23. Mughal S, Filipe MI, Jass JR: A comparative ultrastructural study of hyperplastic and adenomatous polyps, incidental and in association with colorectal cancer. Cancer 1981, 48:2755–2755
24. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, ed 2. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1989
25. Leube RE, Bosch FX, Romano Y, Zimbelmann R, Höfler H, Franke WW: Cytokeratin expression in simple epithelia. III. Detection of mRNAs encoding human cytokeratins nos. 8 and 18 in normal and tumor cells by hybridization with cDNA sequences *in vitro* and *in situ*. Differentiation 1989, 33:69–85
26. Thompson JA: Molecular cloning and expression of carcinoembryonic antigen gene family members. Tumor Biol 1995, 16:10–16
27. Wagener C, Csaszar H, Totovic V, Breuer H: A highly sensitive method for the demonstration of carcinoembryonic antigen in normal and neoplastic colonic tissue. Histochemistry 1981, 58:1–11
28. Wagener C, Müller-Wallraf R, Nissen S, Gröner J, Breuer H: Localization and concentration of carcinoembryonic antigen (CEA) in gastrointestinal tumours: correlation with CEA levels in plasma. J Natl Cancer Inst 1981, 57:539–547
29. Takahashi H, Okai Y, Paxton RJ, Hefta LFH, Shively JE: Differential regulation of carcinoembryonic antigen and biliary glycoprotein by γ-interferon. Cancer Res 1993, 53:1612–1619
30. Prall F, Nollau P, Neumaier M, Haubeck H-D, Drzeniek Z, Helmchen U, Löning T, Wagener C: CD66a (BGP), an adhesion molecule of the carcinoembryonic antigen family, is expressed in epithelium, endothelium, and myeloid cells in a wide range of normal human tissues. J Histochem Cytochem 1996, 44:35–41
31. Stoffel A, Neumaier M, Gaida FJ, Fenger U, Drzeniek Z, Haubeck HD, Wagener C: Monoclonal, anti-domain, and anti-peptides antibodies assign the molecular weight 160,000 antigen of the CD66 cluster to a mRNA encoded by the biliary glycoprotein (BGP) gene, a member of the carcinoembryonic antigen (CEA) gene family. J Immunol 1993, 150:4978–4984
32. Kunath T, Ordonez-Garcia C, Turbide C, Beauchemin N: Inhibition of colonic tumor cell growth by biliary glycoprotein. Oncogene 1995, 11:2375–2382
33. Neumaier M, Fenger U, Wagener C: Monoclonal antibodies for carcinoembryonic antigen (CEA) as a model system: identification of two novel CEA-related antigens in meconium and colorectal carcinoma tissues by Western blots and differential immunoaffinity chromatography. J Immunol 1985, 135:3604–3609
34. Cole JW, McKalen A: Observations of cell renewal in human rectal mucosa *in vivo* with thymidine-H^3. Gastroenterology 1961, 41:122–125
35. Mukherjee TM, Williams AW: A comparative study of the ultrastructure of microvilli in the epithelium of small and large intestine of mice. J Cell Biol 1967, 34:447–461
36. Lorenzsson V, Trier JS: The fine structure of human rectal mucosa. Gastroenterology 1968, 55:88–101
37. Hsieh J-T, Luo W, Song W, Wang Y, Kleinerman DI, Van NT, Lin S-H: Tumor suppressive role of an androgen-regulated epithelial cell adhesion molecule (C-CAM) in prostate carcinoma cell revealed by sense and antisense approaches. Cancer Res 1995, 55:190–197