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Poorly Differentiated Colonic Adenocarcinoma, Medullary Type

Clinical, Phenotypic, and Molecular Characteristics

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Clinicopathological evidence has accumulated that colorectal adenocarcinoma with minimal or no glandular differentiation constitutes two entities with different prognosis. In a series of 20 predominantly nonglandular, poorly differentiated adenocarcinomas, histological features, DNA content, p53 protein expression, Ki-*ras *mutation, and microsatellite instability were analyzed and correlated to the biology of the tumors. In addition, the presence of Epstein-Barr virus (EBV) transcripts was tested by RNA* in situ *hybridization and EBV DNA was demonstrated by nested polymerase chain reaction. Histologically, 13 tumors showed small uniform cells and 7 tumors showed large pleomorphic cells. Tumors with uniform cells exhibited more commonly an expansive growth pattern (69.2%* versus *0%; P < 0.025) and a dense peritumor lymphoid infiltrate (84.6%* versus *14.3%; P < 0.01) resembling their gastric counterpart, solid or medullary carcinoma. These tumors showed less frequent lymph node as well as hematogeneous metastases than pleomorphic carcinomas. In addition, they were usually diploid (84.6%* versus *28.6%; P < 0.05) and lacked stabilization of the p53 protein (0%* versus *42.9%; P < 0.05). No significant difference between the medullary and the pleomorphic tumor type was found with respect to* bcl2 *expression and the occurrence of Ki-*ras *mutations at codon 12. In contrast, microsatellite instability was almost totally restricted to poorly differentiated adenocarcinomas of the medullary type (100%* versus *14.3%; P < 0.001). Finally, polymerase chain reaction revealed EBV DNA in 5 tumor specimens, which was, however, restricted to the peritumor lymphoid infiltrate as shown by* in situ *hybridization. Correlation with the biology of the tumors revealed that only one patient with the uniform cell type died due to metastastic disease during the follow-up period (median, 31 months), which was the case in five of the seven patients with the pleomorphic-type carcinoma (P < 0.025). Our results clearly indicate that the poorly differentiated colonic carcinoma with minimal or no glandular structures constitute two different entities, a medullary and a pleomorphic variant, which markedly differ in their phenotype, genotype, and prognosis. (Am J Pathol 1997, 150:1815–1825)

Colorectal carcinomas with a predominantly nonglandular differentiation form a heterogeneous group of neoplasms that differ in their histological appearance and biological behavior. The differential diagnosis includes poorly differentiated adenocarcinoma, signet-ring-cell carcinoma, and undifferentiated carcinoma. In contrast to the former, the latter tumor type has no glandular structures or other features indicating definitive differentiation.[1]

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As glandular structures or mucin-producing cells can often be demonstrated only after an extensive examination, particularly those tumors with solid growth pattern have been denominated both as uniform undifferentiated carcinomas[2] or as poorly differentiated adenocarcinomas of the medullary type.[3] In the stomach, the term medullary carcinoma has been used for tumors that are considered to be a solid variant of intestinal-type adenocarcinoma.[4] As the morphological features of the nonglandular colorectal cancer with solid growth pattern are identical to their gastric counterparts, we favor the term poorly differentiated colorectal adenocarcinoma of medullary type in case of uniform cytology and of pleomorphic type in case of cellular anaplasia.

There is evidence that these carcinomas represent a heterogeneous group of neoplasms. In two series of eight and seven patients,[2,3] respectively, it has been shown that the prognosis of the so-called medullary type of poorly differentiated adenocarcinoma is better than in the nonmedullary (pleomorphic) type tumors of the colon. These observations raise the question as to the molecular changes underlying these two types of predominantly non-glandular colorectal carcinoma.

Recently, Kim et al[5] found in a study of 137 sporadic colorectal cancers three poorly differentiated adenocarcinomas of medullary type, all of which expressed the so-called replication error phenotype. This type of mutation, characterized by length variations in simple repetitive, microsatellite DNA sequences of normal and tumor DNA (microsatellite instability, MIN), has been described as a novel mechanism in the tumorigenesis of colorectal carcinoma and is closely linked to hereditary non-polyposis cancer (HNPCC).[6–8] Therefore, it is estimated that approximately 5 to 10% of colorectal carcinomas in HNPCC are of the poorly differentiated medullary type.[9] In addition, the morphology of these tumors has on first glance some similarities with the lymphoepithelioma-like carcinoma of the stomach, which is almost invariably associated with Epstein-Barr virus (EBV) infection.[10–12]

To analyze the clinical, immunophenotypic, and molecular characteristics of this tumor type, a series of 20 predominantly nonglandular poorly differentiated colorectal carcinomas was investigated using techniques of flow cytometry, immunohistochemistry, and molecular biology. In particular, we addressed the following questions. First, which are the clinical, histological, and immunohistochemical features most relevant to the diagnosis? Second, which type of genomic instability do these tumors show with respect to ploidy and microsatellite instability? Third, do these tumors show p53 protein stabilization and Ki-*ras* oncogene activation similar to adenocarcinomas? And fourth, is there any association with EBV infection?

Materials and Methods

Patients, Histopathology, and Immunohistology

We investigated a series of 20 poorly differentiated colorectal adenocarcinomas with solid growth pattern from three different hospitals in Germany (Kiel, n = 4; Bamberg, n = 3; Regensburg, n = 13). The most important clinicopathological findings are summarized in Table 1.

Patients 15 to 17 were included in an earlier study and were investigated again with respect to the immunohistological profile, Ki-*ras* mutation at codon 12 and EBV DNA.[13] All patients were adults (age range, 32 to 89 years; mean, 58.2 years). The female to male ratio was 11:9. Follow-up information was available for all patients and lasted from 13 to 55 months (median, 31 months).

Resection specimens were obtained from all patients and were fixed in 10% neutral buffered formaldehyde. Serial sections (4 μm) were cut from paraffin-embedded tissue blocks. The first two sections were stained with hematoxylin and eosin and periodic acid Schiff (PAS) after diastase digestion. The density of the so-called Crohn's-like lymphoid infiltrate surrounding the tumor was graded as proposed by Graham and Appelman[14] on a scale from 0 to 4, with grades 3 and 4 resembling intense lymphoid infiltration. Staging was performed in accordance with the criteria of the International Union Against Cancer (UICC).[15] According to the World Health Organization classification,[1] only those tumors that showed a predominantly solid growth pattern with minimal (<5%) or no glandular differentiation were included. Carcinomatous differentiation was indicated by focal mucin production, shown by PAS-diastase staining. To rule out neuroendocrine small-cell carcinoma, lymphoma, or leukemic deposits, immunohistochemical analyses were carried out on subsequent sections using the streptavidin-biotin-peroxidase complex method with 3,3-diaminobenzidine as brown chromogen. The sections were incubated overnight at 4°C with the primary antisera directed against cytokeratin (clone KL1, Dianova, Hamburg, Germany; 1:100), CEA (DAKO, Hamburg, Germany, 1:600), human leukocyte common antigen (clone PD7/26, DAKO; 1:100), chromogranin A

Table 1. *Clinicopathological Findings of the Patients*

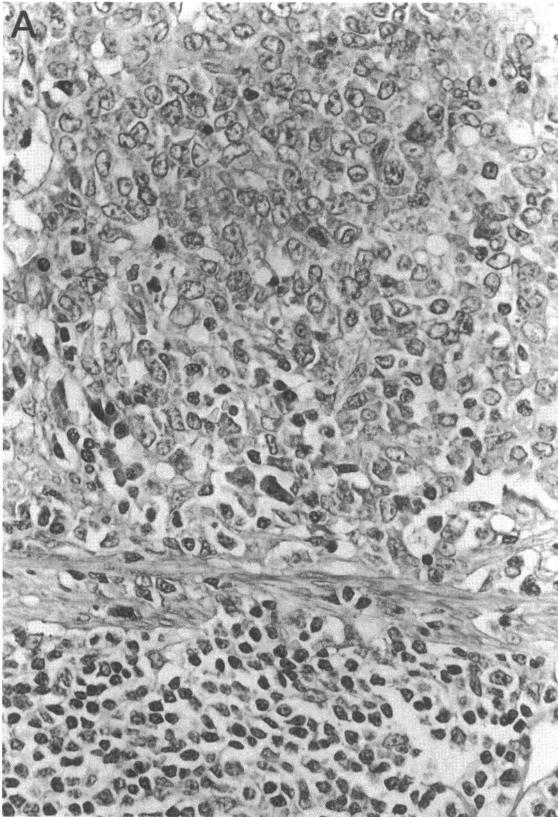
Case	Age (years) Sex	Site	pTNM stage*	Family history	Follow-up	Tumor type†
1	34 M	Ascendens	T4,N2,M0	Sporadic	NED	Uniform
2	49 M	Cecum	T3,N0,M0	HNPCC‡	NED	Uniform
3	78 F	Cecum	T3,N0,M0	Sporadic	DND	Uniform
4	89 F	Cecum	T4,N0,M0	Sporadic	DND	Uniform
5	40 M	Cecum	T3,N0,M0	Sporadic	NED	Uniform
6	32 F	Cecum	T3(3),N0,M0	HNPCC§	NED	Uniform
7	51 M	Right flexure	T4,N0,M0	Sporadic	AWD	Pleomorphic
8	38 M	Recurrent	T1(r),N0,M0	HNPCC‡	NED	Uniform
9	85 F	Cecum	T3,N1,M0	Sporadic	DOD	Pleomorphic
10	68 F	Sigma	T4,N1,M0	Sporadic	NED	Pleomorphic
11	44 F	Ascendens	T4,N3,M1	Sporadic	DOD	Pleomorphic
12	39 M	Rectum	T3,N2,M1	Sporadic	DOD	Pleomorphic
13	59 M	Descendens	T4,N3,M1	Mother died of CRC (87 years)	DOD	Pleomorphic
14	58 F	Ascendens	T3,N2,M1	Sporadic	DOD	Pleomorphic
15	79 F	Cecum	T3,N2,M0	Sporadic	NED	Uniform
16	44 M	Ascendens	T3,N0,M0	Sporadic	NED	Uniform
17	70 M	Cecum	T2,N1,M0	Sporadic	NED	Uniform
18	80 F	Right flexure	T4,N3,M0	Sporadic	NED	Uniform
19	79 F	Transversum	T4,N0,M0	Sporadic	NED	Uniform
20	81 F	Rectum	T2,N0,M0	Sporadic	DOD	Uniform

M, male, F, female, CRC, colorectal cancer; NED, no evidence of disease; AWD, alive with disease; DND, died not of disease; DOD, died of disease.
*According to UICC.[15]
†According to the World Health Organization.[1]
‡HNPCC diagnosis made by proven mismatch repair gene mutation.
§HNPCC diagnosis made according to the Amsterdam criteria.[6,23]

(clone LK2H10, Boehringer Mannheim, Mannheim, Germany; 1:250), synaptophysin (clone SY38, Progen, Heidelberg, Germany; 1:20), and neuron specific enolase (DAKO; 1:100). p53 protein stabilization and bcl2 expression were evaluated with corresponding monoclonal antibodies (clone DO-1 (1:50) for p53; Oncogene Science, Uniondale, NY, and clone 124 for bcl2 (1:60), DAKO) after microwave pretreatment of deparaffinized sections (four times for 4 minutes each at 900 W in 0.1 mol/L citrate buffer). Normal rabbit and mouse sera were used as negative controls. Appropriate positive tissue controls were included for p53 staining in every experiment. In bcl2-stained slides, infiltrating lymphocytes served as internal positive controls. Both p53 and bcl2 immunoreactions were evaluated according to Baas et al[16] with tumors showing high labeling index (LI > 30%), low labeling index (1% < LI < 30%), and no positivity (LI < 1%).

Ploidy Analysis

DNA ploidy was determined by dual-parameter flow cytometry. In the 13 cases from Regensburg, fresh frozen samples were crushed under liquid nitrogen. After two washing steps with phosphate-buffered saline (PBS), the cells were fixed and stored in 70% methanol. Two additional washing steps followed before the cells were incubated with anti-cytokeratin 18 (Boehringer Mannheim) overnight at 4°C, washed three times with 5 ml of PBS, and incubated for 2 hours with the secondary anti-mouse IgG fluorescein-isothiocyanate-conjugated antibody diluted in PBS (1:100). After RNAse A digestion (75 mg/ml RNAse A at 37°C for 30 minutes; Boehringer Mannheim), 10 μg/ml propidium iodide (PI; Sigma, Deisenhofen, Germany) was added on ice for 10 minutes before measurement. Controls were prepared as described above and were stained with the secondary antibody only. Fluorescence of the cells stained with PI and fluorescein-isothiocyanate-conjugated antibodies was measured by means of dual-parameter flow cytometry on a FACScan cytometer (Becton Dickinson, Heidelberg, Germany). In the remaining cases, 50-μm-thick sections from paraffin blocks were deparaffinized in xylene over 24 hours. After rehydration in descending alcohols, the sections were incubated in protease type XXIV (Sigma Chemical Co., St. Louis, MO; 8 U/mg, 5 mg/ml PBS, pH 7.2) for 3 hours. Suspensions were filtered through 40-μm nylon mesh, centrifuged at 2000 × *g*, and resuspended in PBS containing RNAse A (75 mg/ml at 37°C for 30 minutes; Boehringer Mannheim). PI (Sigma, Deisenhofen, Germany; 50 μg/ml) was added on ice for 10 minutes before measurement. DNA ploidy was evaluated with the CellFit software program (Becton Dickinson).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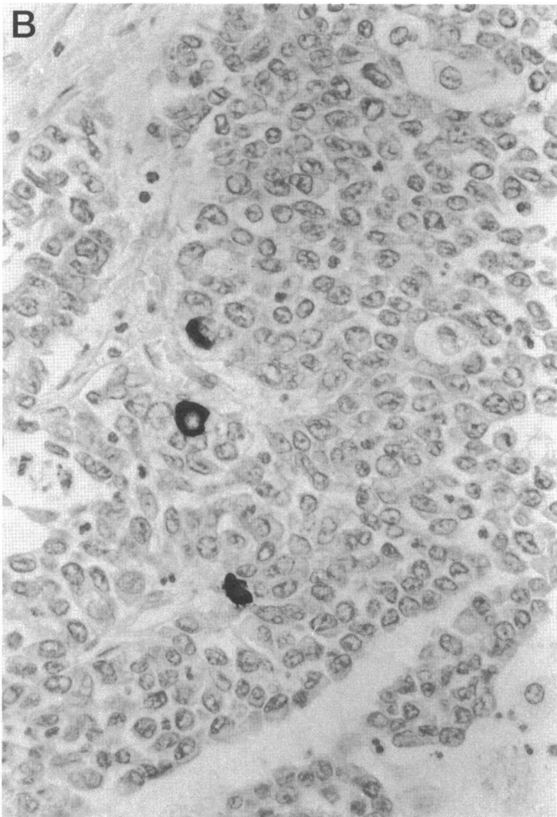
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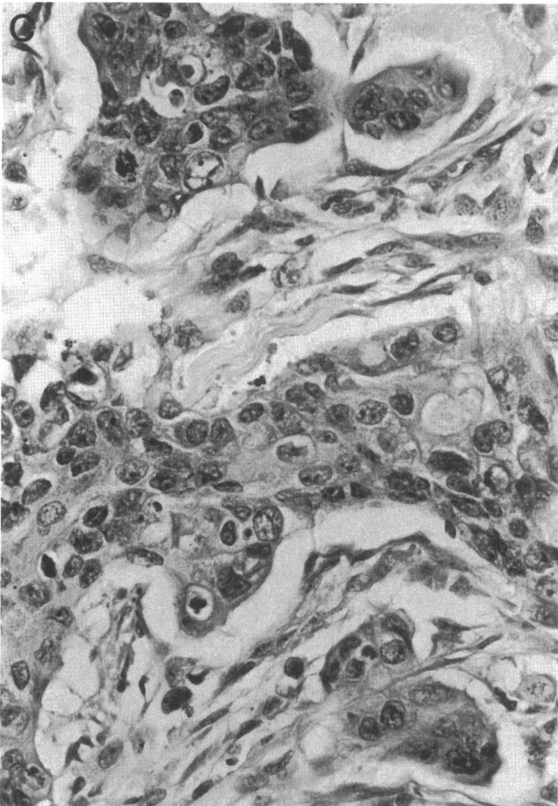
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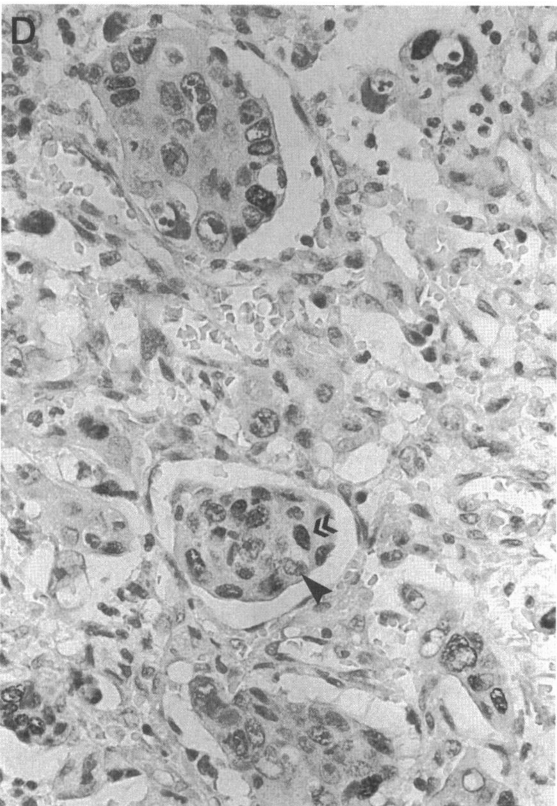
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*DNA Isolation and Ki-*ras *and Microsatellite Analysis*

Genomic DNA was prepared from two to six serial 5-μm-thick paraffin sections after deparaffinization and microdissection of the normal and tumor tissue under light microscopic control. Specimens were digested with proteinase K (4 μg/ml proteinase K, 0.5% Tween 20 in 10 mmol/L Tris/10 mmol/L EDTA, pH 8.0) overnight at 50°C. Proteinase K was inactivated by heating the sample to 94°C for 10 minutes, and 2 μl of the DNA solution was then used as a template for polymerase chain reaction (PCR).

The detection of Ki-*ras* mutations was performed on tumor DNA by enrichment PCR according to Trümper et al.[17] Corresponding normal tissue DNA served as a negative control. Briefly, a 157-bp fragment of the Ki-*ras* gene was amplified with 0.5 μmol/L each of the primer 5′BstNI (5′ ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT 3′) and 3′WT (5′ TCA AAG AAT GGT CCT GCA CC 3′) in a volume of 50 μl for 15 cycles using 1.5 U of *Taq* polymerase (Boehringer Mannheim). A 5-μl aliquot of the PCR product was subsequently digested with 10 U of *Mva*I (Boehringer Mannheim) in a volume of 20 μl for 2 hours at 37°C, resulting in fragmentation of the wild-type and enrichment of the mutated *ras* gene. A 5-μl volume of the digested PCR fragments was used for a second round of PCR in the presence of 0.5 μmol/L each of primers 5′BstNI and 3′BstNI (5′ TCA AAG AAT GGT CCT GGA CC 3′) in a 50-μl volume for 35 cycles. Finally, a 15-μl aliquot was digested with *Mva*I as described above, and 5 μl of this reaction mixture was analyzed on a 12% native polyacrylamide gel.

Microsatellite DNA was examined for genetic alterations at seven separate $(CA)_n$ repeats on the loci D2S123, D5S346, D10S89, D9S171, p53, D18S34, and D18S42 as previously described.[18] Briefly, PCR amplifications were performed with 100 ng of genomic DNA in a reaction mixture containing 0.3 μmol/L each primer, 0.1 mmol/L each dNTP, 50 mmol/L KCl, 10 mmol/L Tris/HCl pH 8.8, 1.5 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, and 2.5 U of *Taq* polymerase (Promega, Madison, WI) in a final volume of 50 μl. The PCR was carried out in an MJ Research thermocycler (MJ Research, Watertown, MA) for 30 cycles of amplification (94°C for 1 minute, 55 to 64°C for 1 minute, and, depending on primer, 72°C for 2 minutes; first denaturation, 94°C for 5 minutes, and final elongation, 72°C for 10 minutes). Subsequently, PCR products were run in denaturing 6.7% polyacrylamide/50% urea gels (1.5 hours at 2000 V and 50°C) in a Sequigene sequencing gel chamber (Biorad, Hercules, CA) and stained with silver nitrate in a special staining tray. Tumors with mobility shifts of the PCR fragments of the tumor compared with normal tissue at two or more microsatellite loci were recorded as MIN+. Allelic losses of bands in tumor DNA were recorded as loss of heterozygosity and were not considered as microsatellite instability.

EBV Analysis

To demonstrate a possible relationship to EBV infection, DNA extracts of tissues were analyzed by nested PCR amplifying a 119-bp fragment of the region in the *Bam*HI-K fragment, near the internal repeat 3 encoding nuclear antigen 1 gene (first upstream primer, 5′ CGA GGG GCC AGG TAC AGG AC 3′; first downstream primer, 5′ GCT CCT GGT CTT CCG CCT CC 3′; nested upstream primer, 5′ CTG GAA ATG GCC TAG GAG AGA AGG 3′; nested downstream primer, 5′ TCG TCC TCG TCC TCT TCC CCG TC 3′). The PCR reaction was performed using 1.25 U of *Taq* DNA polymerase (Boehringer Mannheim), 0.2 mmol/L deoxynucleotides and 0.5 μmol/L primers (35 cycles of 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C). The PCR products were electrophoretically separated by a 4% NuSieve3:1 Agarosegel (FMC BioProducts, Rockland, ME). The presence of EBV early RNA transcripts was investigated by an *in situ* hybridization kit that was applied according to the supplier's recommendations (BioGenex, San Ramon, CA).

Statistical Analysis

All data were subjected to statistical analysis. The Fisher exact test was used instead of χ^2 values when one or more cells in 2 × 2 contingency tables had an expected frequency less than five. The level of significance was set to $P < 0.05$.

Figure 1. *Histological growth pattern and expression of chromogranin A and p53 protein in undifferentiated colorectal carcinoma of uniform* (**A** *and* **B**) *and pleomorphic type* (**C** *and* **D**). **A**: *Undifferentiated carcinoma, medullary type, without gland formation and small uniform tumor cells. Dense lymphoid infiltrate at the expanding tumor margin (bottom).* **B**: *Some tumor cells show immunoreaction to chromogranin A.* **C**: *Undifferentiated carcinoma, pleomorphic type, showing large irregular nuclei, coarse chromatin, atypical mitoses, and an infiltrative growth pattern.* **D**: *Positive immunoreaction to the p53 monoclonal antibody in most tumor cells* (**arrowhead**: *negative;* **double arrowhead**: *positive nuclear staining). Original magnification, ×400.*

Results

Clinicopathological Findings (Table 1)

The feature common to all tumors was minimal acinar differentiation in less than 5% of the section area (n = 5) or no glandular differentiation (n = 15). Two-thirds of the tumors (n = 13) showed a striking uniformity of the cells with small to medium sized nuclei and a prominent nucleolus, scant cytoplasm, and few mitoses. In most of these tumors, the external margin was circumscribed (n = 9, 69.2%) and there was an intense peritumor, Crohn's-like lymphocytic infiltrate (n = 11, 84.6%) resembling poorly differentiated adenocarcinoma of the uniform or medullary type (Figure 1a). In one of these tumors (number 2), a focally (less than 5% of the tumor cells) intense cytoplasmic PAS positivity indicated the presence of signet-ring cells. Immunohistochemically, all tumors stained positive with anti-cytokeratin. In two cases of the uniform type, single tumor cells (less than 5%) expressed chromogranin A, synaptophysin, and neuron specific enolase (cases 2 and 19; Figure 1b). The seven remaining carcinomas exhibited large pleomorphic cells with numerous atypical mitoses, focal tumor necroses, and an infiltrating growth pattern consistent with poorly differentiated carcinoma of pleomorphic or anaplastic type (Figure 1c).

Although both types of carcinomas were mostly located proximal to the splenic flexure (84.6% *versus* 77.1%), the neoplasms of the uniform type showed a predilection for the cecal region (53.8% *versus* 14.3%; $P < 0.1$). The mean age at diagnosis was similar in both groups (58.4 *versus* 57.7 years). The age distribution, however, differed significantly; tumors with uniform histology were more common among patients younger than 50 years of age at the time of diagnosis (n = 6) with a second peak occurring at 75 years of age or older (n = 6). In contrast, patients with the pleomorphic type of carcinoma were mostly 50 to 75 years of age (n = 4; $P = 0.05$). One patient (number 6) had a family history of colon cancer meeting the criteria of the hereditary nonpolyposis colon cancer syndrome (HNPCC). This patient had three synchronous tumors, one adenocarcinoma grade 2 (colon rectum), one mucinous carcinoma (colon transversum), and one poorly differentiated adenocarcinoma with a flat adenoma component in the cecum. Two other patients (numbers 2 and 8) had a family history highly suggestive of a hereditary background. Both patients were younger than 50 years and their fathers died of colorectal cancer at 61 and 56 years, respectively. In one of the two patients (number 8), the tumor included in this series occurred metachronously 7 months after resection of a grade 2 adenocarcinoma of the sigmoid. In contrast to his first tumor, the recurrent carcinoma was solid and showed a flat adenoma in a pattern very similar to that in the HNPCC patient (number 6). At present, mismatch repair gene analysis has been performed with blood samples of two patients. There was one MLH1 point mutation (C→A) leading to termination at codon 252 in patient 2 and a MSH2 mutation with a splice site mutation (AG→AA) at the intron 5/exon 6 transition in patient 8.

Although both types of poorly differentiated adenocarcinoma were staged as pT3 or pT4 at the time of surgery (77% of the uniform and 100% of the pleomorphic type), uniform carcinomas exhibited significantly less frequent lymph node (30.7% *versus* 85.7%; $P = 0.05$) and hematogeneous metastases (0% *versus* 57.1%; $P < 0.01$). Corresponding to these data, patients with medullary-type carcinoma had a more favorable prognosis. Whereas only one patient with uniform tumor cytology died due to metastastic spread, five of seven patients with anaplastic carcinoma died because of metastastic disease ($P < 0.025$). In addition, one of the two patients alive with anaplastic carcinoma had tumor progression.

Ploidy, Immunohistochemical, and Molecular Data (Table 2)

DNA ploidy and PCR analysis revealed a close relationship between histology and the type of genomic instability. According to flow cytometry, 12 of 13 uniform carcinomas were diploid, which was the case in only 2 of the pleomorphic neoplasms ($P < 0.05$). Moreover, all medullary tumors showed MIN in at least two of the seven tested loci: one at six, one at five, six at four, and five at two loci. In contrast, only one pleomorphic tumor showed MIN (pat. no 13) ($P < 0.001$). This tumor showed MIN at three of the tested loci, and the patient died 14 months after surgery due to metastatic disease (Figure 2). In addition, carcinomas of the uniform cell type showed either a weak (1% < LI <30%; n = 3) or no (LI < 1%; n = 10) p53 protein stabilization. In contrast, only two of the pleomorphic-type carcinomas were p53 negative ($P < 0.05$; Figure 1d). Although most of the tumors exhibited a positive bcl2 immunoreaction (LI > 1%; 65%), this was not related to the tumor type or other variables tested. Similarly, Ki-*ras* mutations were almost equally distributed between poorly differentiated adenocarcinomas of the uniform and pleomorphic type (30.8% *versus* 42.8%). EBV-specific

Table 2. *Histopathological, Immunohistological, and Molecular Findings*

Case	Growth	Peritumor inflammation*	Ploidy	p53†	bcl2†	MIN	k-*ras* (wt/mut)	EBV
1	Expansive	Grade 4	Diploid	0	0	+	wt	−
2	Expansive	Grade 3	Diploid	0	0	+	wt	−
3	Infiltrative	Grade 4	Diploid	0	0	+	wt	−
4	Expansive	Grade 4	Diploid	0	0	+	wt	−
5	Expansive	Grade 2	Aneuploid	1	0	+	mut	−
6	Expansive	Grade 3	Diploid	1	2	+	wt	−
7	Infiltrative	Grade 2	Aneuploid	1	2	−	wt	−
8	Expansive	Grade 4	Diploid	0	1	+	wt	−
9	Infiltrative	Grade 4	Diploid	2	2	−	mut	+
10	Infiltrative	Grade 2	Diploid	2	1	−	mut	+
11	Infiltrative	Grade 2	Aneuploid	0	0	−	mut	−
12	Infiltrative	Grade 0	Aneuploid	2	2	−	wt	−
13	Infiltrative	Grade 1	Aneuploid	1	2	+	wt	−
14	Infiltrative	Grade 1	Aneuploid	0	1	−	wt	−
15	Expansive	Grade 4	Diploid	0	0	+	wt	−
16	Expansive	Grade 4	Diploid	0	2	+	mut	+
17	Expansive	Grade 4	Diploid	0	2	+	wt	−
18	Infiltrative	Grade 4	Diploid	0	2	+	mut	+
19	Infiltrative	Grade 2/3	Aneuploid	1	1	+	mut	−
20	Infiltrative	Grade 0	Diploid	0	2	+	wt	+

EBV DNA was demonstrated by nested PCR. +, positive; −, negative; wt, wild type; mut, mutation at codon 12.
*Scoring 0 to 4 according to Graham et al.[14]
†Labeling index 0 to 2 (see Materials and Methods).

DNA could be demonstrated by nested PCR in five tumors (three medullary and two pleomorphic type; Figure 3a). According to *in situ* hybridization, however, EBV early RNA transcripts were restricted to the peritumor lymphocytic infiltrate (Figure 3b).

Discussion

It has been shown that predominantly nonglandular solid colorectal carcinoma may be separated into two types differing in morphology and biology.[2,3] Our study confirms these observations, and in addition clearly shows that poorly differentiated adenocarcinoma medullary and pleomorphic type differ not only in their clinicopathological features but also in their molecular characteristics. Whereas histology and pT stage are highly suggestive of a poor outcome, this is true only in carcinomas with a marked pleomorphic cytology. Poorly differentiated adenocarcinomas with small uniform cells, however, have a much more favorable prognosis. The features most relevant to the diagnosis of the uniform variant are an expanding growth pattern, a dense peritumor lymphoid infiltrate, only a few lymph nodes, and no hematogeneous metastases. These tumors are mostly diploid, show no p53 protein stabilization, and most importantly exhibit widespread genomic alterations, namely, within the microsatellite DNA.

At present, no exact data about the frequency of poorly differentiated colorectal adenocarcinoma, medullary type, are available. Gibbs[2] found 6 cases

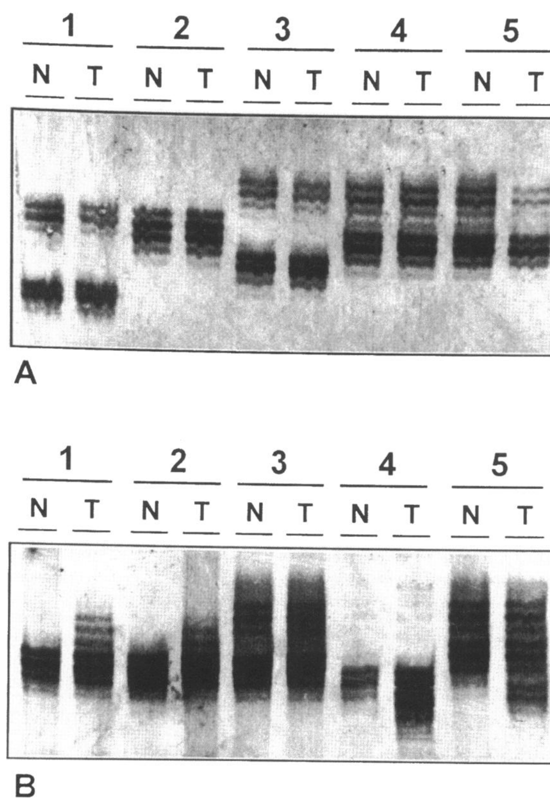

Figure 2. *Microsatellite status at the (CA)$_n$ repeat locus D5S346 in 10 undifferentiated carcinomas.* **A:** *Examples of pleomorphic-type carcinoma with identical allele size of matched normal constitutional (N) and tumor DNA (T). In one tumor (number 5), the intensity of one microsatellite band is reduced, indicating a loss of heterozygosity at the APC gene locus.* **B:** *Examples of uniform-type carcinoma showing additional microsatellite bands in the tumor DNA (T) in cases 1, 2, 4, and 5 indicating microsatellite instability.*

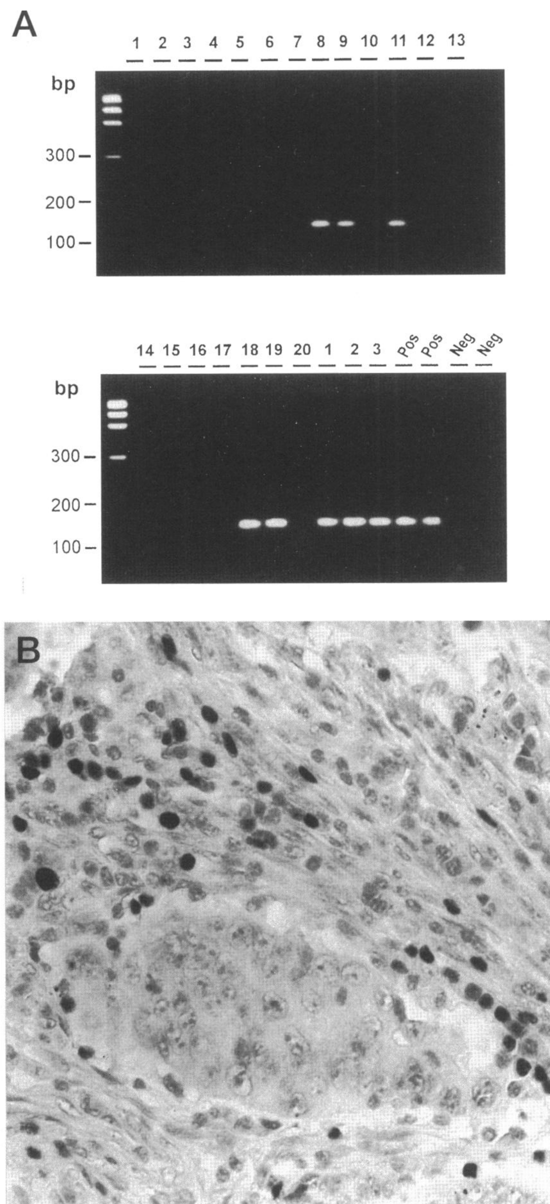

Figure 3. *EBV analysis by nested PCR and in situ hybridization.* **A:** *Detection of EBV-specific PCR products in five cases (8, 9, 11, 18, and 19) of the 20 undifferentiated carcinomas. Three additional medullary gastric carcinomas (1–3) and two EBV-transfected lymphoblastoid cell lines (Pos) served as positive controls (lower gel).* **B:** *EBV early RNA signals are noted by* in situ *hybridization in the nuclei of peritumor lymphocytes but not within the tumor cells. Magnification, ×400.*

of the uniform type among 695 (0.86%) colonic adenocarcinomas at St. Luke's Hospital, London. Although rare, these tumors deserve special interest due to 1) the broad spectrum of differential diagnoses, 2) their clinical course, and 3) the unique molecular changes as shown for the first time in the present study.

The differential diagnosis of solid poorly differentiated colorectal carcinoma comprises undifferentiated carcinoma, signet-ring, and squamous carcinoma as well as small-cell neuroendocrine carcinoma, lymphoma, and leukemic deposits, which may be ruled out by use of mucin stains and immunohistochemical methods. The term undifferentiated carcinoma should therefore be restricted to malignant epithelial tumors that do not show evidence of recognizable differentiation.[1] As most of our cases showed either intracellular PAS material or focal glandular structures, we favor the denomination as poorly differentiated adenocarcinomas, medullary type, in case of uniform cytology, and pleomorphic type in case of anaplastic cytology. Despite the lack of well formed glands or other features indicating unequivocal differentiation, there is growing evidence that at least some forms of poorly differentiated colorectal carcinoma may have a favorable prognosis. Gibbs[2] reported eight cases of uniform undifferentiated carcinomas, of which six patients had a good prognosis; five of these patients survived between 5 and 28 years after surgery. Only two patients with locally irresectable cancer died within 1 year. In another more recent series of seven cases,[3] all patients were alive over a period of 1 to 49 months after tumor resection. The main finding common to both studies was the solid, predominantly nonglandular morphology with a histological appearance mimicking endocrine carcinoma or the medullary variant of gastric cancer. In our study, poorly differentiated adenocarcinomas with and without pleomorphism were included. However, it clearly became evident that only those carcinomas of the uniform or so-called medullary variant have a favorable prognosis. This is in agreement with the abovementioned studies, which included only the medullary variant[3] or excluded the pleomorphic type from their series.[2]

The features most relevant for the diagnosis of the uniform variant of poorly differentiated adenocarcinoma is the uniformity of cells, the expansive growth pattern without obvious necroses, and most remarkably a dense peritumor lymphoid infiltrate. PAS stains revealed focal intracellular mucin at least in some cells in most cases. In one case, there was a clear signet-ring differentiation in approximately 5% of the tumor (number 2). This tumor, together with one other carcinoma (number 19) showed also a focal neuroendocrine differentiation. These features were restricted to the uniform variant and are similar to those noted by Gibbs,[2] who found argyrophil cells in four of his tumors. In addition, only one of his tumors showed lymph node and hepatic metastases. This is very similar to our data where lymph node and hematogeneous metastases were significantly less frequent in the medullary than in the pleomorphic tumor type.

Evidence of a possible premalignant lesion was found in two of our cases. Both patients had a positive family history of colon cancer, which was consistent with the diagnostic criteria of HNPCC in one patient (number 5). In the second patient (number 8), a hMSH2 mismatch repair gene mutation could be demonstrated. In both tumors, a flat adenoma next to the infiltrative undifferentiated carcinoma was visible. As flat adenomas may represent an attenuated variant of familial adenomatous polyposis coli,[19] there is at present no general agreement about the frequency of flat adenoma in HNPCC. Our data confirm, however, at least some of the more recent studies in which flat adenomas have been found, particularly in kindreds with the Lynch (HNPCC) syndrome.[20,21] These precursor lesions have a much higher potential for malignant transformation than polyps of similar size,[22] which might explain the very short time interval between the development of carcinomas (within 2 to 3 years[23]) in HNPCC patients. Regarding the bimodal age distribution in the medullary-type carcinomas, it is tempting to speculate that the older patients have truly sporadic cancers whereas the younger ones are hereditary. The latter represent either new mutations or patients whose family histories are incomplete or too subtle to be diagnosed by Amsterdam criteria.

These unique findings, particularly with respect to clinical course, histology, and premalignant lesions in poorly differentiated adenocarcinoma prompted us to study the molecular changes in more detail. For the first time, we could demonstrate that the two types of poorly differentiated adenocarcinoma differ significantly in their DNA content, p53 protein expression, and microsatellite status. The medullary-type carcinomas were mostly diploid and did not show overexpression of the p53 protein. Most interestingly, instabilities in the microsatellite DNA were in all but one case restricted to the uniform medullary variant of poorly differentiated adenocarcinoma ($P < 0.001$). Microsatellite instability, as evidenced by expansions or reductions in the length of short tandem repeats (microsatellites) between constitutive and tumor DNA, is now widely accepted as an indicator for a new mechanism of carcinogenesis. In approximately 80% of colorectal carcinomas, an accumulation of particularly severe mutations has been described that leads to aneuploidy and loss of heterozygosity in tumor suppressor genes such as APC, DCC, and p53.[24] In contrast, approximately 15 to 20% of sporadic and approximately 85 to 90% of HNPCC-related colorectal carcinomas show MIN or replication errors.[5–7] These neoplasms lack the gross genomic lesions such as loss of heterozygosity at the various tumor suppressor genes and are mostly diploid.[25] Recently, we have confirmed these findings by using comparative genomic hybridization. Only MIN-negative tumors showed gross genomic lesions.[26] These genomic changes occur primarily in HNPCC-related carcinomas due to mutations in the human homologues of prokaryotic mismatch repair genes (hMSH2, hMLH1, hPMS1, and hPMS2; for review see Ref. 27). The clinicopathological features that have been identified in sporadic colorectal carcinomas expressing MIN are similar to the features of colorectal cancers in HNPCC patients. Both show a propensity for the proximal colon, a tendency toward younger patient age, and an excess of mucinous and signet-ring-cell-type carcinomas.[5] Most interestingly, it has been noticed that approximately 5 to 10% of HNPCC carcinomas exhibit a unique morphology with solid growth pattern resembling poorly differentiated adenocarcinoma of medullary type.[9] This is in accordance with our study in which one patient with the uniform carcinoma type had a HNPCC family history and two others had a proven mismatch repair gene mutation.

The occurrence of MIN in sporadic poorly differentiated adenocarcinomas of the medullary type was first noticed by Kim et al[5] in three cases. Similar to our series, all three tumors exhibited an intense peritumor lymphoid reaction. The presence of lymphoid infiltrates at the advancing tumor margin has been introduced as an independent prognostic factor, initially in rectal carcinomas by Jass[28] and later also in colorectal carcinomas.[14] Thereby, it has been shown that colorectal tumors with a distinct so-called Crohn's-like lymphoid reaction are more often right-sided advanced carcinomas. From the molecular point of view, there is growing evidence that MIN might also be the major contributing factor to the intense peritumor host immune reaction. Cancer cells with widespread mutations in the microsatellite DNA express a particular variety of mutated proteins that could raise a protective immune response.[29]

As the histological pattern of the uniform poorly differentiated colorectal adenocarcinoma has at first glance some similarities with lymphoepithelioma-like gastric carcinoma, we tested also the presence of EBV. EBV is directly involved in a variety of neoplastic disorders, notably nasopharyngeal carcinoma, Burkitt's lymphoma, and Hodgkin's disease. Recently, EBV DNA has been demonstrated in most of the gastric carcinomas with lymphoid stroma.[11,12] The similar morphology between these tumors and medullary colorectal carcinomas has prompted the search for EBV. To our knowledge, this is the first report in which EBV has been studied within poorly

differentiated colorectal adenocarcinoma. Although EBV DNA could be demonstrated in five tumor specimens by nested PCR, *in situ* hybridization revealed EBV-specific RNA transcripts of EBV early RNA type only within the tumor-associated lymphoid infiltrate. In contrast to the lymphoepithelioma-like gastric carcinomas, we therefore have no evidence of an association between the medullary poorly differentiated colorectal carcinoma and EBV infection. These findings basically confirm that, at least in the colorectum, solid medullary adenocarcinoma should not be mixed up with the lymphoepithelioma-like carcinoma. In the latter cases, the entire tumor tissue is diffusely infiltrated by lymphoid cells, generally with infiltrating growth pattern at the advancing edges. These tumors are extremely rare in the colon. In contrast, the solid or medullary-type adenocarcinoma shows closely packed cells with a well defined boundary. The lymphocytic infiltrate is rather peritumoral, forming the so-called Crohn's like lesion.[1,4,13] Finally, similar to gastric carcinomas, we found no significant relationship between EBV infection and MIN status.[30]

In conclusion, our data strongly support the view that poorly differentiated colorectal adenocarcinomas form two different groups of distinct clinicopathological and molecular characteristics. The pleomorphic variant follows the classical type of carcinogenesis with accumulation of gross genomic lesions, cytological pleomorphism, and an aggressive clinical behavior. In contrast, the medullary variant shows multiple small genomic lesions in the microsatellites, with uniform histology and a good prognosis.

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