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Keratin 20 - A diagnostic and prognostic marker in colorectal cancer?

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Summary. Colorectal cancer cells characteristically show strong expression of keratin 20 (K20) and lack expression of keratin 7 (K7). The biological significance of reduced K20 expression, however, is unclear. 381 colorectal cancers with 148 corresponding metastases were evaluated for K20 and K7 expression by immunohistochemistry using a tissue microarray technique. K20 immunoreactivity was assessed semiquantitatively as either negative, low (<50% of cancer cells) or high (≥50% of cancer cells). Progression-free and cancer-specific survivals were determined using the Kaplan-Meier method. Expression of K20 was observed in 348 out of 372 (94%) evaluable primary tumors, with 135 (36%) cases showing low K20 and 213 (57%) cases high K20 expression, while 24 (6%) tumors completely lacked K20 immunoreactivity. Reduced K20 expression (lack of staining or low expression) was significantly associated with poor differentiation, large tumor size and mismatch repair deficiency, but did not significantly affect patients' outcome. Immunoreactivity of K20 and K7 in metastatic tissues matched well with that of corresponding primary tumors, with high concordance for lymph node ($p<0.001$) and distant metastases ($p<0.001$), respectively. In conclusion, our data illustrate the value of keratin subtyping in carcinoma of unknown primary (CUP) syndrome: K20 expression is common in colorectal cancer and the K20 high / K7 negative immunoprofile represents the predominant phenotype. Reduced K20 expression may, however, lead to false-negative assessment of metastatic deposits if only small amounts of tissue are obtained (e.g. in needle biopsies), particularly in poorly differentiated cancers. Reduced expression of K20 may be used to select tumors for microsatellite instability testing.

Key words: Colorectal carcinoma, Keratin 20, Keratin 7, Carcinoma of unknown primary, Prognosis

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Introduction

The simple epithelial intermediate filament keratin 20 (K20) is characteristically present in non-neoplastic epithelium of the colon and rectum and is constitutively expressed in carcinomas arising from these sites. Hence, K20 plays a crucial role in immunoprofiling of colorectal carcinomas (CRC), including both primary and metastatic cancer tissues. Particularly, when dealing with carcinomas of unknown primary (CUP), in which histopathology alone may be inconclusive, keratin subtyping may help to identify CRC origin of metastatic deposits with possible clinical implications for the selection of chemotherapy regimens (Dennis et al., 2005; Varadhachary et al., 2008). Currently, the most commonly applied multi-marker profile to distinguish CRC from other cancers combines K20 with keratin 7 (K7), another simple epithelial intermediate filament, as well as the nuclear transcription factor CDX-2 (Caudal-type homeobox 2) (Dennis et al., 2005; Bahrami et al., 2008; Lugli et al., 2008).

Expression of K20 in CRC ranges from 68% to 100% of cases (Wang et al., 1995; Chu et al., 2000; Kummar et al., 2002; Lassmann et al., 2002; Park et al., 2002; Kende et al., 2003; Dennis et al., 2005; Hernandez et al., 2005). Moreover, K20 positive / K7 negative is the predominant immunoprofile in CRC and accounts for 68% to 95% of tumors (Chu et al., 2000; Park et al., 2002; Kende et al., 2003; Hernandez et al., 2005; Lugli et al., 2008). Nevertheless, in a minority of cases alteration of the epithelial cytoskeleton may occur during neoplastic transformation and/or cancer

progression.

In a previous study we were able to demonstrate that acquisition of K7 expression in CRC prevails in budding cancer cells at the leading front of invasion. Of note, patients with K7 positive CRC were more likely to experience disease progression compared with patients with K7 negative tumors, but data just missed statistical significance (Harbaum et al., 2011). According to data in the literature, loss of K20 may represent a marker for progression in CRC (Hernandez et al., 2005; Bressenot and Zimmer, 2008). Likewise, Lugli et al. (2008) noted K20 expression to be an independent adverse prognostic factor in mismatch repair (MMR) proficient tumors. However, data on the prognostic impact of reduced K20 expression, as well as that of different K20 / K7 immunoprofiles in an unselected cohort of CRC patients are currently lacking.

The aim of our current study was to correlate K20 expression and K20 / K7 immunoprofiles with different pathological parameters, including tumor stage and grade, tumor border configuration and budding, vascular invasion and MMR protein status, and to correlate immunoreactivity in primary tumors with that of corresponding metastases. In addition, we evaluated the prognostic significance of K20 expression and that of different K20 / K7 immunoprofiles, regarding both progression-free and cancer-specific survival.

Material and Methods

Patient selection

During the period from January 1, 1984 through December 31, 2005, a total of 7909 colorectal cancers from 7564 patients (4095 males, 3469 females; ratio 1.2:1) were identified in the CRC database of the Institute of Pathology, Medical University of Graz, Austria. Of these, 400 (5%) patients, operated upon between January 1992 and December 2000, were sampled randomly and included in the investigation. This time-period was chosen to obtain identical adjuvant treatment modalities (see below) as well as at least 5 years of follow-up.

The following patients were excluded: (i) those who underwent endoscopic polypectomy for low-risk T1 cancer due to missing data regarding nodal status; (ii) patients who underwent neoadjuvant chemotherapy due to presumptive treatment-related changes in morphology, including changes in TNM classification; (iii) patients with synchronous or metachronous secondary colorectal cancer; and (iv) patients with competitive invasive cancers originating from other sites if metastatic deposits were not assessed by histology.

In total, 381 specimens from 400 patients (95%) were available for review pathology. There were 215 males (56%) and 166 females (44%) (ratio 1.3:1) with a median age of 68.5 (range 27.6-93.1) years. Of these, 191 (50%) of 381 were older than 70 years. Adjuvant chemotherapy was adjusted to N classification. Patients with node-negative tumors did not receive chemotherapy, whereas patients with node-positive tumors or with tumor progression were given chemotherapy according to the Mayo regimen (5-FU plus leucovorin) (Moertel et al., 1990).

All patients had laboratory checks every 3 months (including blood count, liver enzymes and tumor markers CEA and CA 19-9); after 3 years the interval was extended to 6 months. Chest X-ray and ultrasonography of the abdomen were obtained at 6-month intervals; after 3 years the interval was extended to 12 months. Patients with rectal cancer had a yearly computerized tomography (CT) scan of the abdomen and pelvis.

Institutional review board approval was received from the Ethic's Committee of the Medical University of Graz, Austria.

Histopathology

Review-pathology of primary tumors and corresponding metastatic tissues (if present) was performed independently by two investigators (MP and CL). Discrepancies were resolved by simultaneous re-examination of the slides by both investigators using a double-headed microscope. T and N classification were adjusted to the AJCC/UICC TNM system (Sobin and Wittekind, 2002). Histological tumor type and tumor grades were assessed according to the WHO classification (Hamilton et al., 2000).

Presence of lymph and/or blood vessel invasion was assessed carefully. Only vessels with an unequivocal endothelial lining were considered true lymphatic vessels. Special care was taken to differentiate endothelial cells from retraction artifacts lined by fibroblasts. When carcinoma was present in vessels with a thick vascular wall and red blood cells in the lumen, this was considered blood vessel invasion. Tumor budding was defined as the presence of isolated single cells or small clusters of cells (composed of fewer than five cells) scattered in the stroma at the invasive tumor margin. The extent of tumor budding was assessed as described previously in a field in which budding intensity was maximal using a x20 objective lens (Ueno et al., 2002). The number of budding foci was counted as follows: score 1 (<5 budding foci), score 2 (5-9 budding foci), score 3 (10-19 budding foci), and score 4 (≥20 budding foci). Tumors were then divided into two groups according to the number of budding foci: counts of 0-9 were termed low-grade, while counts of 10 or more foci were termed high-grade budding.

Immunohistochemistry

For immunohistochemical evaluation a tissue microarray (TMA) technique was used. The details of this technique have been described previously (Kononen et al., 1998). Briefly, TMAs were constructed using a manual tissue arraying instrument (Beecher, Silver

Spring, MD, USA). To account for tumor heterogeneity, between 3 and 14 (mean 5.03, median 5) cylindrical core biopsies, 0.6mm in diameter, were taken from different sites of each tumor and arrayed in a recipient paraffin TMA block (Fig. 1). Corresponding lymph node and distant metastases were included in 143 and 42 cases, respectively. Four micrometer TMA sections were stained using an automated staining system (Dako-Autostainer, Universal Staining System; Dako, Glostrup, Denmark).

Antibodies directed against K20, K7, as well as the MMR proteins MLH1, MSH2 and MSH6 were included in the investigation. Table 1 lists all used commercially available antibodies, including dilution and epitope retrieval method, as well as the detection system applied. Non-neoplastic epithelium served as internal positive control for K20 staining, and slides of pancreatic adenocarcinoma as positive control for K7 staining. Negative controls included omission of the primary antibody and incubation with Dako REAL™ Antibody Diluent (Code S2022; Dako). For the MMR proteins intratumoral lymphocytes served as positive control. Negative controls included omission of the primary antibody and incubation with Dako REAL™ Antibody Diluent (No. S2022, Dako) or Ventana Antibody Diluent (Ventana, Tucson, AZ, USA ; Catalog No. 251-018), respectively.

Immunoreactivity was assessed independently by two investigators (LH and CL), who were blinded to clinicopathological data. Discrepancies were resolved by simultaneous re-examination of the slides by both investigators using a double-headed microscope. A distinct cytoplasmic or membranous staining for K20 was considered positive. K20 immunoreactivity was semiquantitatively categorized as "low" (<50% of tumor cells positive), "high" (≥50%) or "negative". Expression of K7 was assessed as "positive" or "negative". According to Lugli et al. (2008) for a tumor to be considered mismatch repair (MMR) protein proficient, immunoreactivity for hMLH1, hMSH2 and hMSH6 was required, whereas loss of immunoreactivity for at least one of the three markers characterized MMR deficient tumors. Any loss of MMR protein expression on microarray analysis was validated by analysis of whole sections. Similarly, in case of equivocal staining on microarray sections, whole sections were used to define MMR protein status.

Statistical analysis

Associations of K20 expression and the K20 / K7 immunoprofile with conventional tumor parameters were analyzed using Chi-square or Fisher's exact test, respectively. Progressive disease was defined as either local recurrence (any detectable local disease at follow-up, occurring either alone or in conjunction with generalized recurrence) or systemic recurrence (as any detectable disease at follow-up, except local disease). Cause of death was determined by treating physicians and/or by chart review and was corroborated by death certificates if available. Progression-free (disease-free) and cancer-specific survival was assessed with the Kaplan-Meier method and compared by the log-rank test. To assess concordance of immunostaining results

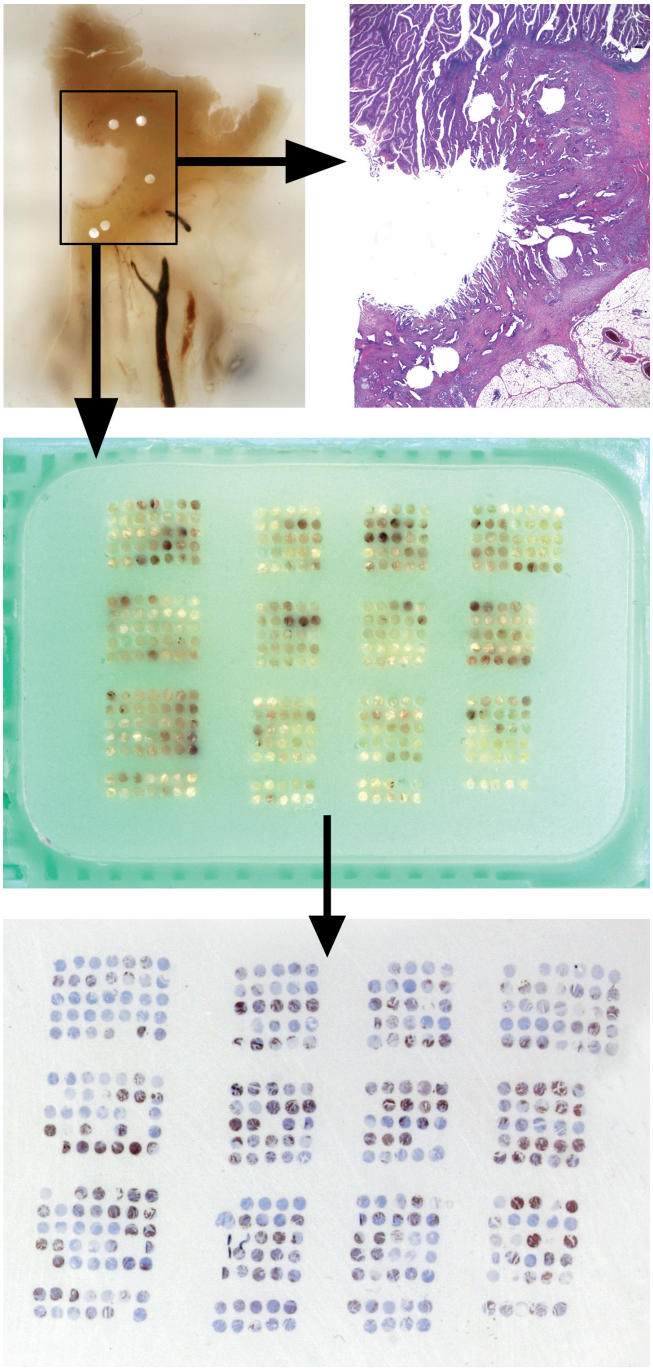

Fig. 1. Construction of a TMA block by taking cylindrical core biopsies, 0.6 mm in diameter, from different sites of a given tumor (top). Keratin 20 immunostaining of the corresponding TMA section (bottom).

between primary and corresponding lymph node and/or distant metastases the Somer's D rank-order correlation coefficient was used. The correlation of K20 / K7 immunoprofiles of primary tumors with those of metastatic sites was performed on tumors with corresponding metastases (case-matched correlation). All reported p-values were 2-sided with significance at $p<0.05$. All statistical calculations were performed using NCSS (Hintze, 2007).

Results

Tumor characteristics

Tumors were located in the caecum in 47 (13%) patients, in the ascending colon in 26 (7%), at the hepatic flexure in 18 (5%), in the transverse colon in 13 (3%), at the splenic flexure in 13 (3%), in the descending colon in 15 (4%), in the sigmoid colon in 80 (21%), at the rectosigmoid junction in 15 (4%), and in the rectum in 147 (39%) patients, respectively. Median tumor size was 4.5 cm (mean 4.7; range 0.6-15).

Overall, 316 (83%) tumours were adenocarcinomas, 45 (12%) mucinous adenocarcinomas, and 13 (3%) undifferentiated carcinomas. Seven cases presented rare histological subtypes, including three signet-ring cell, two medullary, one adenosquamous, and one mixed endocrine-exocrine (composite) carcinoma, respectively. With respect to pT classification, 28 (7%) tumors were classified pT1, 70 (18%) pT2, 218 (57%) pT3, and 65 (17%) pT4. Lymph node metastasis was detected in 168 (44%) cases, wherein 83 (22%) were classified N1 and 85 (22%) N2. Tumor grades were G1 in 121 (32%), G2 in 138 (36%), G3 in 99 (26%) and G4 in 23 (6%) cases. Thus, carcinomas were low grade (G1 and G2) in 259 (68%) cases and high grade (G3 and G4) in 122 (32%) cases. Lymph vessel and blood vessel invasion were detected in 126 (33%) and 87 (23%) cases.

Immunohistochemistry

Expression of K20 was observed in 348 out of 372 (94%) evaluable primary tumors, with 135 (36%) cases showing low K20 and 213 (57%) cases high K20 expression (Fig. 2A,B). 24 (6%) cases lacked K20 immunoreactivity. K20 expression was observed in 115 out of 131 (88%) corresponding lymph node metastases (Fig. 2C,D) and in 33 out of 38 (87%) corresponding distant metastasis. Expression of K7 was observed in 32 out of 370 (9%) evaluable primary tumors, in 11 out of 131 (8%) lymph node metastases and four out of 38 (11%) distant metastasis. The K20 / K7 immunoprofile was assessable in 369 primary tumors, wherein K20 high / K7 negative was found in 194 (53%) cases, K20 high / K7 positive in 17 (5%) cases, K20 low or negative / K7 negative in 144 (39%) cases, and K20 low or negative / K7 positive in 14 (4%) cases.

Expression of K20 was significantly associated with tumor differentiation, tumor size, tumor location, histological subtype, lymphatic invasion, and MMR protein status (Table 2). The K20 / K7 immunoprofile was significantly associated with tumor differentiation, histological subtype and MMR protein status (Table 3). Immunoreactivity of K20 and K7 in metastatic tissues matched well with that of corresponding primary tumors (Table 4, Fig. 2C-F)). Regarding K20, Somer's D coefficients for concordance of primary tumors with corresponding lymph node and distant metastases were D=0.7136 (95% confidence interval [CI] 0.6042 to 0.8231; $p<0.001$) and D=0.75 (95% CI 0.5687 to 0.9313; $p<0.001$). Regarding K7, Somer's D coefficients for concordance of primary tumors with corresponding lymph node and distant metastases were D=0.8453 (95% CI 0.6756 to 1.0000; $p<0.001$) and D=0.6389 (95% CI 0.1760 to 1.0000; $p=0.0119$). Likewise, K20 / K7 immunoprofiles of metastatic tissues matched well with those of corresponding primary tumors (Table 5), with Somer's D coefficients for concordance of primary tumors with corresponding lymph node and distant metastases of D=0.632 (95% CI 0.5024 to 0.7616; $p<0.001$) and D=0.6886 (95% CI 0.4907 to 0.8866; $p<0.001$), respectively.

Survival analysis

Follow-up data were available for 350 (92%) patients. Median follow-up was 45 months (mean 56, range 0-180). At the time of last follow-up, 173 (49%) patients showed no evidence of disease. Progressive disease was observed in 141 (40%) patients including 117 (33%) patients who died from cancer and 11 (3%) patients who currently are alive with metastatic disease.

Table 1. Antibodies used for immunohistochemical staining.

Antibody	Source	Clone	Dilution / Epitope Retrieval	Detection system	Chromogen
K7	Dako (Glostrup, Denmark)	OV-TL 12/30	1:100 / P	A	AEC
K20	Dako	Ks 20.8	1:100/ P	A	AEC
MLH1	Biocare (Concorde, CA, USA)	G168-15	1:50 / MW, Buffer pH 9.0	B	DAB
MSH2	Ventana, (Tucson, AZ, USA)	G219-1129	1:50 / Buffer CC1 standard	C	DAB
MSH6	Biocare	BC-44	1:50 / Buffer CC1 mild	C	DAB

A: Dako REAL Detection System K5001; B: Dako EnVision+ (HRP rab/mouse) K5007; C: Ventana UltraView DAB 760-500; AEC: Aminoethylcarbazole (Dako, S2367); DAB: Diaminobenzidine (Dako, K5001); P: Protease Type XXIV Digestion (Sigma Aldrich, St. Louis, MO, USA, P8298); MW: Microwave; Buffer pH 9.0: Target Retrieval Solution (Dako, S2367); CC1: Ventana (950-124 SL)

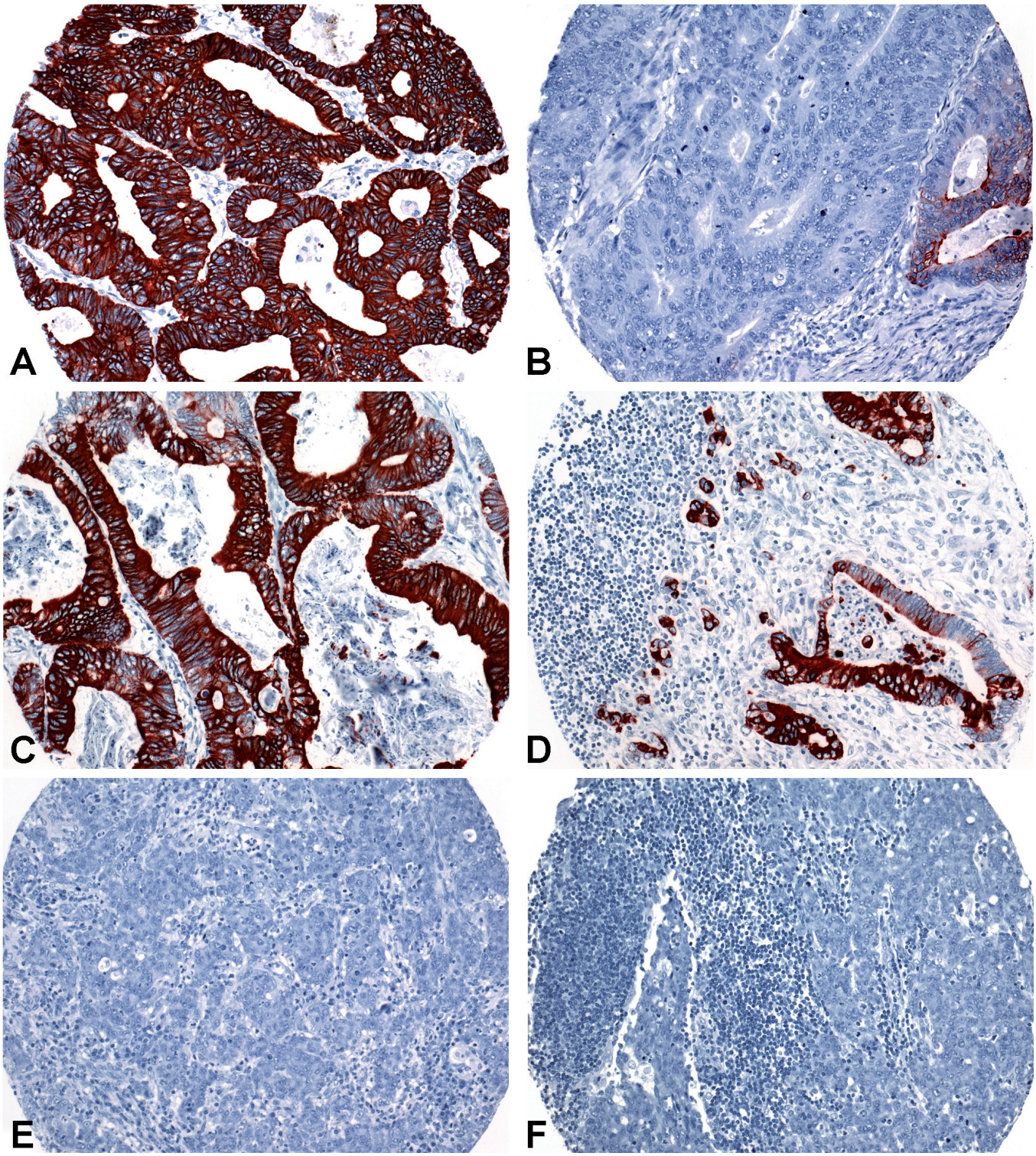

Fig. 2. Expression of keratin 20 (K20): Primary colorectal adenocarcinomas showing high (**A**; ≥50%) and low (**B**; <50%) K20 expression. Diffuse K20 expression in primary colorectal adenocarcinoma (**C**) and corresponding metastastic lymph node deposit (**D**). Negative staining for K20 in primary colorectal adenocarcinoma (**E**) and corresponding metastastic lymph node deposit (**F**). x 200

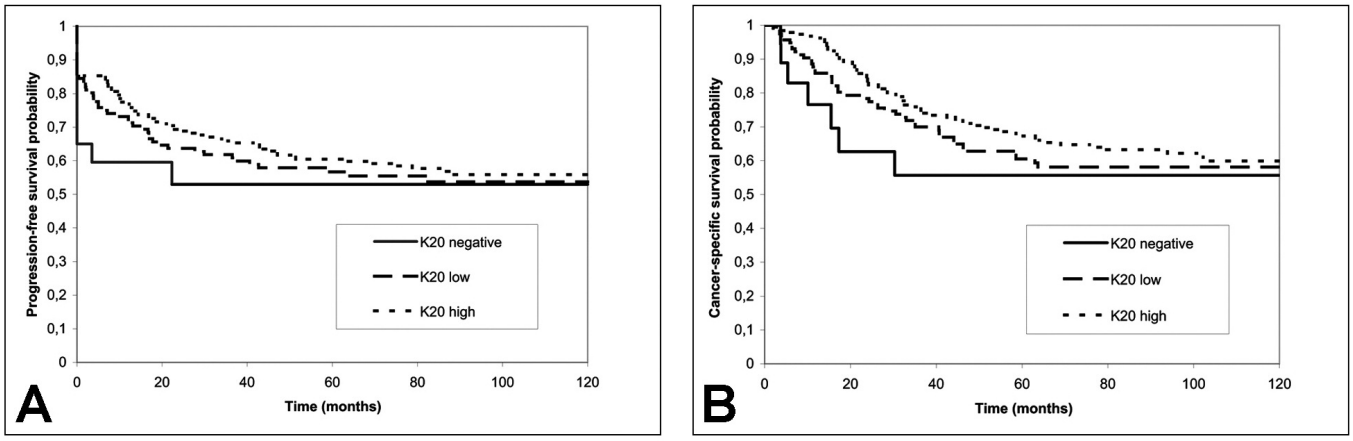

Fig. 3. Progression-free (**A**; p=0.55, log-rank test) and cancer-specific (**B**; p=0.4, log-rank test) survival in patients with colorectal cancer stratified for keratin 20 (K20) expression (Kaplan-Meier univariate analysis).

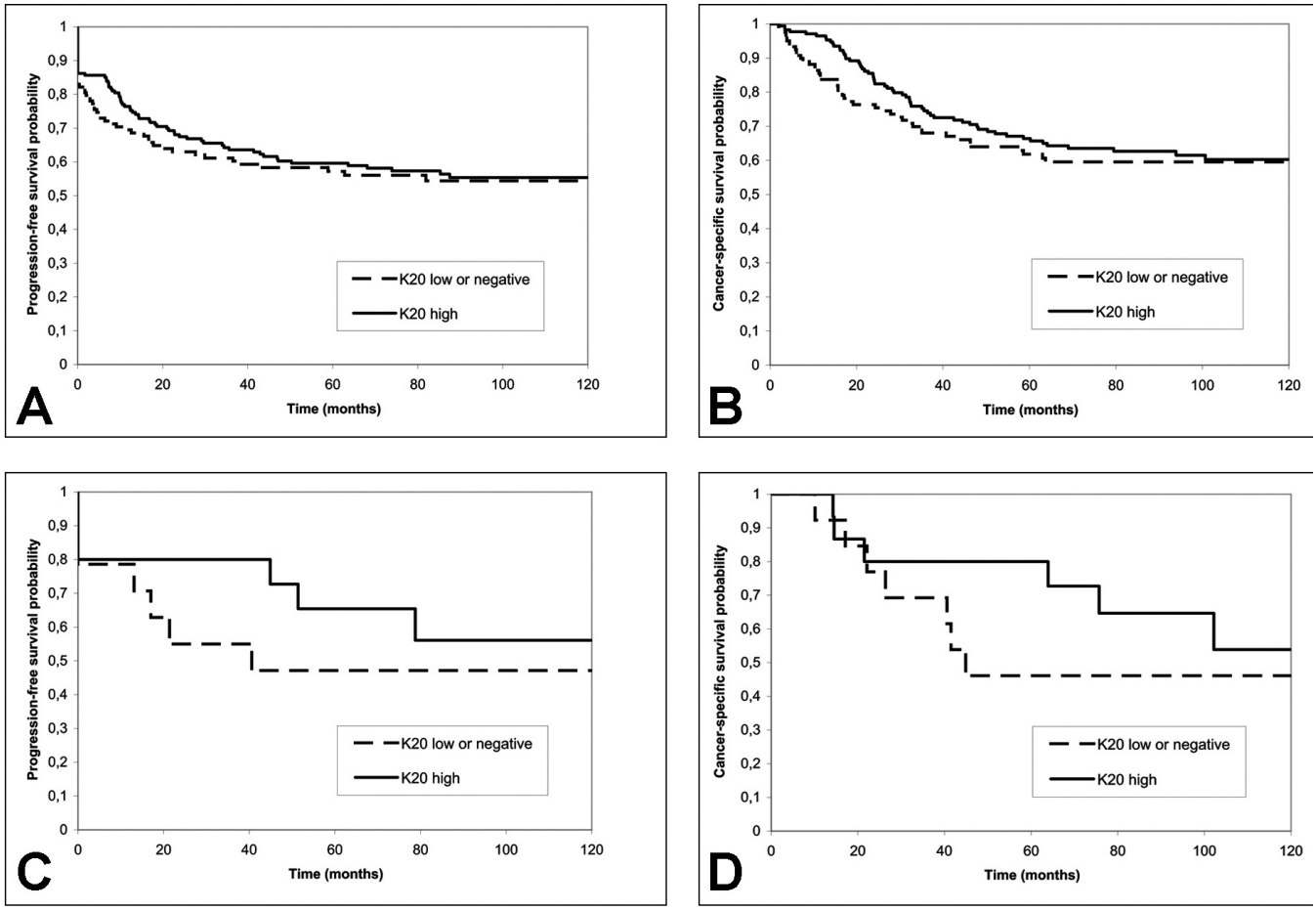

Fig. 4. Progression-free (**A**; p=0.53, log-rank test) and cancer-specific (**B**; p=0.38, log-rank test) survival in patients with keratin 7 (K7) negative colorectal cancers related to keratin 20 (K20) expression. Progression-free (**C**; p=0.42, log-rank test) and cancer-specific (**D**; p=0.39, log-rank test) survival in patients with K7 positive colorectal cancers related to K20 expression (Kaplan-Meier univariate analysis).

Median time to progression was 7 months (mean 15, range 0-88). 49 (14%) patients died from causes not related to CRC.

Disease progression occurred in 80 out of 198 (40%) patients with tumors showing high K20 expression, 51 out of 123 (41%) patients with tumors showing low K20 expression, and 9 out of 20 (45%) patients with tumors lacking K20 expression (p=0.55, log-rank test; Fig. 3A). Actuarial 5-year progression-free survival rates for patients with tumors showing high K20 expression, low K20 expression, and lack of K20 expression were 60%, 57% and 53%, respectively.

In addition, 66 out of 198 (33%) patients with tumors showing K20 high expression, 44 out of 123 (36%) patients with tumors showing K20 low expression and 7 out of 20 (35%) patients with tumors lacking K20 expression died of disease (p=0.40, log-rank test; Fig. 3B). Actuarial 5-year cancer-specific survival rates for patients with tumors showing K20 high expression, patients with tumors showing K20 low expression and patients with tumors lacking K20 expression were 68%, 61% and 56%, respectively.

Of note, however, Kaplan-Meier curves diverged markedly within the first two years after surgery, eventually converging after approximately five years. Thus, K20 expression may influence the date of progression, but may not determine whether progression occurs or not. Therefore, we decided to analyze survival rates after two years and noticed a significant difference regarding cancer-specific survival (p=0.028, log-rank test): 29 out of 198 (15%) patients with tumors showing high K20 expression, 24 out of 123 (20%) patients with tumors showing low K20 expression, and 6 out of 20 (30%) patients with tumors lacking K20 expression died of disease within two years after surgery.

When cancers were stratified for K7 expression, K20 showed no prognostic significance, regarding both progression-free and cancer-specific survival in K7-negative (Fig. 4A,B) and K7-positive (Fig. 4C-D) tumors, respectively. Furthermore, when UICC stage II and stage III cancers, and tumors showing MMR protein proficiency or deficiency were analyzed separately, K20 expression had no effect on patients' survival, regardless of whether tumors were additionally stratified for K7 expression (data not shown).

Table 2. Association of keratin 20 (K20) expression with different tumor characteristics.

	K20 negative (n=24)	K20 low (n=135)	K20 high (n=213)	p-value
T classification				0.393
T1	-	7 (5%)	19 (9%)	
T2	3 (13%)	25 (19%)	40 (19%)	
T3	15 (63%)	75 (56%)	123 (58%)	
T4	6 (24%)	28 (21%)	31 (15%)	
N classification				0.328
N0	10 (42%)	79 (59%)	118 (53%)	
N1	5 (21%)	26 (19%)	51 (24%)	
N2	9 (37%)	30 (22%)	44 (21%)	
UICC stage				0.362
I	2 (8%)	28 (21%)	49 (23%)	
II	7 (29%)	45 (33%)	64 (30%)	
III	8 (33%)	44 (33%)	72 (34%)	
IV	7 (29%)	18 (13%)	28 (13%)	
Tumor grade				0.001
Low	9 (37%)	89 (66%)	156 (73%)	
High	15 (63%)	46 (31%)	57 (27%)	
Histology				<0.001
Adenocarcinoma	18 (75%)	111 (82%)	182 (85%)	
Mucinous CRC	1 (4%)	15 (11%)	26 (12%)	
Undifferentiated CRC	5 (21%)	5 (4%)	2 (1%)	
Other CRC	-	4 (3%)	3 (1%)	
Tumor size				0.006
≤4.5 cm	7 (30%)	64 (50%)	122 (61%)	
>4.5 cm	16 (70%)	64 (50%)	77 (39%)	
Tumor location				0.04
Right	11 (46%)	43 (32%)	52 (24%)	
Left	9 (37%)	37 (27%)	60 (28%)	
Rectum	4 (17%)	55 (41%)	101 (48%)	
Tumor budding				0.812
Low	13 (54%)	76 (56%)	126 (59%	
High	11 (46%)	59 (44%)	87 (41%)	
Lymphatic invasion				0.025
L0	10 (42%)	93 (69%)	146 (69%)	
L1	14 (58%)	42 (31%)	67 (31%)	
Venous invasion				0.06
V0	14 (58%)	104 (77%)	167 (79%)	
V1	10 (42%)	31 (23%)	46 (21%)	
MMR protein status				<0.001
Proficient	16 (67%)	123 (91%)	209 (99%)	
Deficient	8 (33%)	12 (9%)	3 (1%)	

Discussion

Keratin subtyping represents a well established diagnostic tool in primary and metastatic carcinoma tissues. It reflects the general observation that tumors tend to recapitulate the keratin profile of their non-neoplastic cell lineage. CRC characteristically show expression of K20 and lack expression of K7 (Wang et al., 1995; Chu et al., 2000). However, according to our data, approximately 40% of primary CRC show reduced K20 expression (6% of tumors lacking K20 expression, 36% of tumors showing low K20 expression). These results are fairly in line with previous literature data, wherein K20 negative CRC accounts for 0% to 32% of cases (Wang et al., 1995; Chu et al., 2000; Kummar et al., 2002; Lassmann et al., 2002; Park et al., 2002; Kende et al., 2003; Dennis et al., 2005; Hernandez et al., 2005). In addition, semiquantitative analysis of K20 expression as performed by immunohistochemistry or conventional reverse transcriptase polymerase chain reaction (RT-PCR) was shown to be well reproducible by quantitative realtime RT-PCR (Lassmann et al., 2002).

In a previous study, we demonstrated that approximately 10% of CRC acquire K7 expression during the neoplastic process (Harbaum et al., in press).

K7 expression was mainly focal with less than 10% of cancer cells stained and was associated with poor tumor differentiation and a high degree of tumor budding. Furthermore, patients with K7 positive CRC were more likely to experience disease progression, but data just missed statistical significance (p=0.06).

In our present analysis, reduced K20 expression occurred more frequently in tumors with poor

Table 3. Association of keratin 20 (K20) / keratin 7 (K7) immunoprofile with different tumor characteristics.

		K20 negative or low		K20 high		p-value
		K7 negative (n=144)	K7 positive (n=14)	K7 negative (n=194)	K7 positive (n=17)	
T classification	T1	7 (5%)	-	18 (9%)	1 (6%)	0.5
	T2	26 (18%)	2 (14%)	36 (19%)	3 (17%)	
	T3	79 (55%)	10 (71%)	110 (57%)	12 (70%)	
	T4	32 (22%)	2 (14%)	30 (15%)	1 (6%)	
N classification	N0	82 (57%)	7 (50%)	107 (55%)	9 (53%)	0.856
	N1	29 (20%)	2 (24%)	47 (24%)	4 (23%)	
	N2	33 (23%)	5 (36%)	40 (21%)	4 (23%)	
UICC stage	I	28 (19%)	2 (14%)	45 (23%)	3 (17%)	0.882
	II	49 (34%)	3 (21%)	57 (29%)	6 (35%)	
	III	46 (32%)	5 (36%)	66 (34%)	6 (35%)	
	IV	21 (15%)	4 (29%)	26 (13%)	2 (12%)	
Tumor grade	Low	90 (63%)	7 (50%)	147 (76%)	7 (41%)	0.002
	High	54 (37%)	7 (50%)	47 (24%)	10 (59%)	
Histology	Adenocarcinoma	117 (81%)	11 (79%)	169 (87%)	11 (65%)	0.015
	Mucinous CRC	15 (10%)	1 (7%)	21 (11%)	5 (29%)	
	Undifferentiated CRC	8 (6%)	2 (14%)	2 (1%)	-	
	Other CRC	4 (3%)	-	2 (1%)	1 (6%)	
Tumor size	≤4.5 cm	64 (47%)	7 (50%)	111 (61%)	9 (60%)	0.094
	>4.5 cm	72 (53%)	7 (50%)	71 (39%)	6 (40%)	
Tumor location	Right	49 (34%)	5 (36%)	48 (25%)	4 (23%)	0.051
	Left	45 (31%)	1 (7%)	56 (29%)	2 (12%)	
	Rectum	50 (35%)	8 (57%)	90 (46%)	11 (65%)	
Tumor budding	Low	83 (58%)	5 (36%)	117 (60%)	7 (41%)	0.158
	High	61 (42%)	9 (54%)	77 (40%)	10 (59%)	
Lymphatic invasion	L0	96 (67%)	7 (50%)	134 (69%)	11 (65%)	0.521
	L1	48 (33%)	7 (50%)	60 (31%)	6 (35%)	
Venous invasion	V0	109 (76%)	9 (54%)	152 (78%)	13 (76%)	0.663
	V1	35 (24%)	5 (36%)	42 (22%)	4 (24%)	
MMR protein Status	Proficient	127 (88%)	11 (79%)	192 (99%)	16 (94%)	<0.001
	Deficient	17 (12%)	3 (21%)	2 (1%)	1 (6%)	

Table 4. Concordance of keratin 20 (K20) and keratin 7(K7) expression in primary and corresponding metastatic tumor tissues (n=148 primary tumors with lymph node (n=131) and/or distant (n=38) metastases).

Primary Tumor	Lymph Node Metastasis		Distant Metastasis	
K20 negative 12/148 (8%)	Negative	9/12 (75%)	Negative	4/4(100%)
	Low	1/12 (8%)	Low	-
	High	-	High	-
K20 low 53/148 (36%)	Negative	4/49 (8%)	Negative	1/13 (8%)
	Low	30/49 (61%)	Low	10/13 (77%)
	High	15/49 (31%)	High	2/13 (15%)
K20 high 83/148 (56%)	Negative	1/70 (1%)	Negative	-
	Low	4/70 (6%)	Low	4/21 (19%)
	High	65/70 (93%)	High	17/21 (81%)
K7 positive 13/148 (9%)	Negative	2/11 (18%)	Negative	1/3 (33%)
	Positive	9/11 (82%)	Positive	2/3 (67%)
K7 negative 135/148 (91%)	Negative	121/122 (99%)	Negative	35/36 (97%)
	Positive	1/122 (1%)	Positive	1/36 (3%)

differentiation and large tumor size, tumors located within the right hemicolon, and in MMR protein deficient tumors. These observations are well in line with previous literature data, stating reduced K20 immunoreactivity to be more frequent in poorly differentiated (Park et al., 2002; Kende et al., 2003; Lugli et al., 2008), right sided (Park et al., 2002), and MMR protein deficient tumors (McGregor et al., 2004; Lugli et al., 2008). In addition, loss of K20 expression has been significantly associated with high AJCC/UICC stage (Hernandez et al., 2005) and presence of intratumoral lymphocytes (Lugli et al., 2008). In our cohort, CRC with reduced K20 expression similarly tended to occur at high AJCC/UICC stage, although this association lacked statistical significance. The association with tumor dedifferentiation and with increasing tumor stage indicates gradual loss of K20 during cancer progression. Remarkably, Tatsumi et al. (2006) noted that reduced K20 expression is already observed during the early steps of cancer formation with markedly reduced expression in malignant tissues compared with colorectal adenomas.

The K20 positive / K7 negative immunoprofile marks the predominant phenotype in CRC, accounting for 68% to 95% of tumors (Chu et al., 2000; Park et al., 2002; Kende et al., 2003; Hernandez et al., 2005; Lugli et al., 2008). Likewise, we found K20 high / K7 negative to be the most frequent phenotype. Alterations in the K20 / K7 immunoprofile were significantly associated with high tumor grade and MMR protein deficiency, and occurred more often within the right hemicolon, although this association missed statistical significance. Previous studies noted altered K20 / K7 immunoprofiles to be more frequent in high grade tumors (Park et al., 2002; Kende et al., 2003), within the right hemicolon (Park et al., 2002), and, additionally, in tumors with high AJCC/UICC stage (Hernandez et al., 2005). Of note, McGregor et al. (2004) observed no difference of K20 / K7 immunoprofiles between microsatellite stable and unstable CRC.

As indicated above, keratin profiles may change during tumor initiation and progression. Importantly, we present the first systematic study investigating the concordance between primary CRC and corresponding metastatic tissues with respect to keratin staining. According to our data, the expression profile of metastatic tissue matches well with that of corresponding primary tumors, regarding K7, K20, and also regarding K20 / K7 immunoprofiles. Our data thus substantiate the value of keratin subtyping in cases with CUP syndrome (Tot, 1999; Park et al., 2007; Bahrami et al., 2008). However, if only small amounts of tissue are obtained (e.g. in needle biopsies), reduced or heterogeneous K20 expression may lead to false-negative assessment of cancer tissue (Tot and Samii, 2003; Goldstein and Bosler, 2007), and pathologists need to be aware of this potential pitfall.

Although several studies addressed the topic of K20 expression in CRC, little is known about its prognostic significance. In our analysis, the expression of K20, regardless of its extent, did not significantly affect patients' outcome. However, disease progression occurred earlier in patients with tumors showing reduced K20 expression compared with patients with tumors showing high (i.e., normal) K20 expression. Thus, reduced expression of K20 in CRC may indicate a high propensity for short-time recurrence after surgery. The reason for this observation is unclear, and future studies are warranted to address this topic.

Currently, there is only one further study available addressing the prognostic significance of K20 expression in CRC. Remarkably, Lugli et al. (2008) stated high (i.e., normal) K20 expression, which was termed K20 overexpression by the authors, to be an independent adverse prognostic factor in MMR protein proficient, yet not in deficient tumors. The authors speculated that the high amount of intratumoral lymphocytes they observed in K20 negative tumors may account for the more

Table 5. Concordance of combined keratin 20 (K20) and keratin 7 (K7) immunoprofiles in primary and corresponding metastatic tumor tissues (n= 148 primary tumors with lymph node (n=131) and/or distant (n=38) metastases).

Primary Tumor	Lymph Node Metastasis		Distant Metastasis	
K20 low or negative / K7 negative 59/148 (40%)	K20 low or - / K7-	37/40 (67%)	K20 low or - / K7-	12/14 (86%)
	K20 low or - / K7+	-	K20 low or - / K7+	1/14 (7%)
	K20 high / K7-	13/40 (33%)	K20 high / K7-	1/14 (7%)
	K20 high / K7+	-	K20 high / K7+	-
K20 low or negative / K7 positive 6/148 (4%)	K20 low or - / K7-	2/5 (40%)	K20 low or - / K7-	1/2 (50%)
	K20 low or - / K7+	2/5 40%)	K20 low or - / K7+	1/2 (50%)
	K20 high / K7-	-	K20 high / K7-	-
	K20 high / K7+	1/5 (20%)	K20 high / K7+	-
K20 high / K7 negative 75/148 (51%)	K20 low or - / K7-	11/68 (16%)	K20 low or - / K7-	4/19 (21%)
	K20 low or - / K7+	-	K20 low or - / K7+	-
	K20 high / K7-	57/68 (84%)	K20 high / K7-	14/19 (74%)
	K20 high / K7+	-	K20 high / K7+	1/19 (5%)
K20 high / K7 positive 8/148 (5%)	K20 low or - / K7-	-	K20 low or - / K7-	-
	K20 low or - / K7+	-	K20 low or - / K7+	-
	K20 high / K7-	1/8 (13%)	K20 high / K7-	-
	K20 high / K7+	7/8 (87%)	K20 high / K7+	2/2 (100%)

favorable prognosis. Therefore, we separately analyzed the influence of K20 expression in MMR protein proficient and deficient tumors, although K20 expression did not significantly affect outcome in these subgroups.

There are several limitations to our study. First and foremost are the limitations inherent to retrospective analyses. By excluding patients with polypectomized low-risk T1 cancer and missing data regarding nodal status, patients with neoadjuvant chemotherapy, patients with synchronous or metachronous secondary CRC, as well as patients with competitive invasive cancers originating from other sites if metastatic deposits were not assessed by histology, we tried to control the homogeneity of the study population. Nevertheless, the patients included in this study underwent surgical therapy by multiple surgeons from both academic and community settings.

In conclusion, K20 is common in CRC and the K20 high / K7 negative immunoprofile marks the predominant phenotype with high concordance between primary and corresponding metastatic tumor tissues. Reduced K20 expression was significantly associated with poor differentiation, large tumor size, and MMR protein deficiency, but did not significantly affect patients' outcome.

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