Differential diagnostic and functional role of the multi-marker phenotype CDX2/CK20/CK7 in colorectal cancer stratified by mismatch repair status

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The differentiation of colorectal cancer from primary tumors at other sites can be challenging. Often a panel of immunohistochemical protein markers is needed to distinguish between these entities. Protein expression differs significantly in colorectal cancer depending on mismatch repair status and is also heterogeneous among mismatch repair-proficient or -deficient tumors. The aim of this study was to systematically analyze the diagnostic and prognostic role of the commonly used multi-marker phenotype CK20/CK7/CDX2 on a large series of colorectal cancers stratified by mismatch repair status. The immunohistochemical analysis of CK20, CK7 and CDX2 was performed on 1197 mismatch repair-proficient and 223 mismatch repair-deficient colorectal cancers using a tissue microarray. Multi-marker combinations of CK20/CK7/CDX2 were explored. Univariate and multivariable analysis of the markers was evaluated for their association with several clinico-pathological end points namely T stage, N stage, tumor grade, vascular invasion, intratumoral lymphocytes and survival. Multi-marker phenotypes with CK20 and CDX2 negativity were more frequently found in mismatch repair-deficient than in mismatch repair-proficient colorectal cancer (19.3 vs 7.5% and 21.6 vs 6.7%, respectively; $P < 0.001$). In both colorectal cancer subsets loss of CK20 was associated with higher tumor grade ($P < 0.001$) and with presence of intratumoral lymphocytes ($P < 0.001$ and $P = 0.02$, respectively). In the proficient mismatch repair subset CK20 overexpression was an independent adverse prognostic factor ($P = 0.041$) and CDX2 under-expression was linked to tumor progression. Loss of CDX2 and CK20 is more frequently encountered in mismatch repair-deficient colorectal cancer, which should be taken into consideration to differentiate between primary and metastatic colorectal cancer in daily practice. Although associated with lower tumor grade, CK20 overexpression is an independent adverse prognostic factor in mismatch repair-proficient colorectal cancer. *Modern Pathology* (2008) 21, 1403–1412; doi:10.1038/modpathol.2008.117; published online 27 June 2008

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In diagnostic surgical pathology one of the most important goals is the correct assignment of tumor from a preoperative biopsy and can be complicated by the common practical problem of the ‘unknown primary cancer’. Particularly, the primary site of a metastatic carcinoma can be very difficult to determine by morphology alone.^{1–4}

Immunohistochemical markers are a well established tool in daily diagnostic practice and help to define tumor lineage when antibodies are applied against specific tumor antigens.⁵ In particular, the

differential expression profile of various cytokeratin (CK) types by tumors at different primary locations can be essential in determining the origin of a malignant tumor.^{1,2,4,6–8}

CKs are intermediate filament proteins and 20 subtypes have been classified based on isoelectric pH (focusing) and molecular weight.^{5,9–11} Two of the most used stains are CK20 and CK7.^{5,10,12,13} CK20 is normally expressed in the gastrointestinal epithelium, Merkel cells and urothelium^{14,15} whereas CK7 can be detected in normal tissue and tumors of the lung, breast, ovary, biliary tract and endometrium.^{2,5} According to several studies the multi-marker phenotype CK20+/CK7– is observed in colorectal cancer in approximately 75–95%^{2,14} of cases whereas the multi-marker phenotype CK20–/CK7+ favors primary tumors of the lung, breast, biliary tract, pancreas, ovary and endometrium.⁵ However, the combined use

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of CK20 and CK7 is limited, especially in the distinction of primary and secondary mucinous tumors in the ovary¹⁶ thereby making the addition of other markers mandatory.

CDX2 is a cloned caudal-type homebox gene encoding a nuclear transcription factor involved in proliferation and differentiation of intestinal epithelial cells.^{17,18} Although CDX2 expression is observed with high frequency in colorectal cancer (61–100%), the broad range of CDX2 expression in primary ovarian mucinous tumors (0–100%) decreases the diagnostic utility of CDX2 for distinguishing colorectal cancer from primary ovarian mucinous tumors.¹⁹

The differentiation between colorectal cancer and primary tumors at other sites is additionally complicated by the fact that colorectal cancers are a heterogeneous group of tumors. Indeed, sporadic colorectal cancers have been classified into two main groups, namely those with chromosomal instability evidenced by loss of heterozygosity as well as DNA aneuploidy and linked with mutation of *APC*, *KRAS* and *TP53*²⁰ and those with DNA microsatellite instability associated with infrequent loss of heterozygosity and diploid DNA. Colorectal cancer with microsatellite instability has been linked with *BRAF* mutation and methylation of the DNA mismatch repair gene *MLH1*.^{21,22} Current research supports the notion of multiple pathways in colorectal cancer defined by combined molecular features such as mismatch repair, CpG island methylator phenotype and O6-methyl-guanine-DNA methyltransferase status as well as *KRAS* and *BRAF* mutation.²³

Certain clinico-pathological features such as tumor-infiltrating lymphocytes, proximal subsite, mucinous histology, poor differentiation, Crohn-like reaction and diagnosis before the age of 50 years seem to predict microsatellite instability high status.^{24–32} Although a reduced CK20 expression in microsatellite instability high colorectal cancer was described in a previous study,³³ the immunohistochemical phenotype of microsatellite instability high colorectal cancer is poorly studied.

On the basis of the above described morphological and immunohistochemical profiles we hypothesized that the use of the multi-marker phenotype CK20/CK7/CDX2 can be a potential pitfall to distinguish a primary colorectal cancer from other tumor entities, for example, a metastasis of a mucinous ovarian tumor. Therefore, the aim of the present study was to analyze the diagnostic and functional role of the multi-marker phenotype CK20/CK7/CDX2 on a large series of mismatch repair-proficient ($n=1197$) and mismatch repair-deficient colorectal cancer ($n=223$).

Materials and methods

Tissue Microarray Construction

A tissue microarray of 1420 unselected, non-consecutive colorectal cancer was constructed as

described previously.³⁴ Formalin-fixed, paraffin-embedded tissue blocks of colorectal cancer resections were retrieved from the archives of the Institute of Pathology, University Hospital of Basel, Switzerland, the Institute of Clinical Pathology, Basel Switzerland and the Institute of Pathology, Stadtspital Triemli, Zürich, Switzerland. One-tissue cylinder with a diameter of 0.6 mm was punched from morphologically representative tissue areas of each 'donor' tissue block and brought into one-recipient paraffin block (3 × 2.5 cm) using a homemade semiautomated tissue arrayer. Failure of analysis (<10%) was related to tissue microarray technology including missing samples or fractions containing only a few tumor cells.

Normal Colon Tissue

In addition to colorectal cancer, 57 normal colon tissues were integrated into the tissue microarray and were used as a control group to compare expression of protein markers.

Immunohistochemistry of Tissue Microarray

Immunohistochemistry was performed for protein markers MLH1, MSH2, MSH6, CD8, CK20, CK7 and CDX2. Briefly, four-micrometer sections of tissue microarray blocks were transferred to an adhesive-coated slide system (Instrumedics Inc., Hackensack, NJ, USA) to facilitate the transfer of tissue microarray sections to slides and to minimize tissue loss. Standard indirect immunoperoxidase procedures were used for immunohistochemistry (ABC-Elite, Vector Laboratories, Burlingame, CA, USA). All 1420 colorectal cancer and 57 normal colonic mucosa samples were immunostained for CK20 (clone Ks20.8, 1:50, DAKO, Baar, Switzerland), CK7 (clone OV-TL 12/30, dilution 1:200, DAKO, Baar, Switzerland), CDX2 (clone AMT28, 1:50, Abcam, Cambridge, UK), MLH1 (clone MLH-1; dilution 1:100; BD Biosciences Pharmingen, San Jose, CA, USA), MSH2 (clone MSH-2; dilution 1:200; BD Biosciences Pharmingen, San Jose, CA, USA), MSH6 (clone 44; dilution 1:400; BD Biosciences Pharmingen, San Jose, CA, USA) and CD8 (clone C8/144B, dilution 1:100; Dako Cytomation, Carpinteria, USA). After dewaxing and rehydration in dH₂O, sections for immunostaining were subjected to heat antigen retrieval in a microwave oven (1200 W, 15 min) in 0.001 mol/l ethylenediaminetetraacetic acid, pH 8.0 for MLH1 and MSH2, pH 9 for CK20 and CK7 and in 0.01 mol/l citrate buffer, pH 6.0 for MSH6 and pH 2 for CDX2. Endogenous peroxidase activity was blocked using 0.5% H₂O₂. After transfer to a humidified chamber, the sections were incubated with 10% normal goat serum (Dako Cytomation) for 20 min and incubated with primary antibody at room temperature for 1 h (CK20, CK7 and CDX2). Subsequently, the sections were incubated with peroxidase-labeled

polymer (K4005, EnVision + System-HRP(AEC); DakoCytomation) for 30 min at room temperature. For visualization of the antigen, the sections were immersed in 3-amino-9-ethylcarbazole + substrate-chromogen (K4005, EnVision + System-HRP (AEC); DakoCytomation) for 30 min, and counterstained lightly with Gill's hematoxylin.

Mismatch Repair Status and Clinico-Pathological Data

Immunohistochemistry was used to identify mismatch repair-proficient and mismatch repair-deficient cases. For a tumor to be considered mismatch repair-proficient, positive expression (>0% tumor cell staining) for MLH1, MSH2 and MSH6 was required. All 1197 cases were determined to be mismatch repair-proficient. The remaining 223 tumors were defined as microsatellite instability high and showed absence of expression of either nuclear MLH1 or MSH2 or MSH6. These microsatellite instability high cases included both sporadic MLH1-negative tumors ($n = 141$) and 82 presumed HNPCC cases with loss of MSH2 and/or MSH6 at any age or loss of MLH1 at <55 years.³⁵ In addition, clinico-pathological data for all patients included age, tumor diameter, tumor location and complete follow-up as well as pT and pN stage, tumor grade and the presence of vascular invasion which were all assessed pathologically from the corresponding surgical resection specimen. In addition, disease-specific survival time was available for 1410 patients.

Evaluation of Immunohistochemistry

All cases were scored by one experienced pathologist (AL). Cases where the interpretation of the immunohistochemical analysis was difficult were discussed with a second experienced pathologist (LT). Cytoplasmic and/or membranous CK20 and CK7 and nuclear CDX2 were scored as the number of positive tumor cells. To consider a case-positive standard assessment of >0% of tumor cells was assigned when analyzing the diagnostic role of these markers and multi-marker phenotypes. To evaluate their prognostic effect and role in tumor progression, cutoff scores for positivity were optimized using receiver operating characteristic (ROC) curve analysis. Intraepithelial CD8+ intratumoral lymphocytes located in direct contact with tumor cells were quantified over the area of the entire punch for each case in the tissue microarray. Tumor in at least 50% of the tissue microarray punch was necessary for the case to be considered evaluable. Staining intensity was not evaluated.³⁶

Statistical Analysis

Differences in mean CK20, CK7 and CDX2 protein expression between mismatch repair-proficient

and -deficient colorectal cancers were obtained by the Wilcoxon test. To determine the association of positive protein expression with clinico-pathological features, cutoff scores for CK20, CK7, and CDX2 expression were determined by ROC curve analysis.³⁷ The ROC curve is a plot of the sensitivity and (1-specificity) for an outcome at each value of the protein expression score. A protein score can therefore be selected from the curve such that a cutoff at this value leads to the greatest number of patients correctly classified as with (maximizing sensitivity) and without (maximizing specificity) the clinical end point. The (0, 1)-criterion was used to minimize the trade-off between sensitivity and specificity. To determine the reliability of the ROC curve-derived cutoff scores for CK20, CK7 and CDX2, 100 bootstrapped replications of the data were performed to re-sample the data. The most frequently obtained cutoff score was selected as the final score above which tumors should be considered positive for the outcome. ROC curve analysis and bootstrapping was repeated for each clinico-pathological end point. The χ^2 and Fisher's Exact Test were used to determine the association between negative and positive CK20 and CK7 expression with T stage, N stage, tumor grade, vascular invasion and tumor location, whereas the Kaplan-Meier method and log-rank test were used for univariate survival analysis. Cox proportional hazards regression was carried out for multivariable survival analysis. Hazard ratios (HR) and 95% confidence intervals (CI) were obtained. P -values <0.05 were considered statistically significant. All analyses were carried out using SAS V.9 (The SAS Institute, Cary, NC, USA).

Results

Mismatch Repair Status

Mismatch repair-deficient tumors compared with mismatch repair-proficient cases displayed the typical characteristics such as preponderance for females (65.0 vs 50.1%; $P < 0.001$), more frequently located in the right side (65.0 vs 29.1%; $P < 0.001$) and more poorly differentiated (22.3 vs 11.0%; $P < 0.001$) (Table 1). In addition, both 5- and 10-year survival rates are significantly improved in mismatch repair-deficient colorectal cancer compared with their mismatch repair-proficient counterparts ($P < 0.001$). These findings thus confirm the representativeness of mismatch repair-deficiency and -proficiency in our patient cohort. Median follow-up time was 46.0 months.

Normal Colonic Mucosa

In normal colonic mucosa CX20, CK7 and CDX2 expression could be detected in 98.4, 0 and 100% of cases, respectively.

Table 1 Characteristics of patients with mismatch repair-proficient and -deficient colorectal cancer

Characteristic	Frequency N (%)	
	Mismatch repair-deficient	Mismatch repair-proficient
Gender		
Male	78 (35.0)	595 (49.9)
Female	145 (65.0)	596 (50.1)
Tumor location		
Left-sided	78 (35.0)	834 (70.9)
Right-sided	145 (65.0)	343 (29.1)
pT stage		
pT1	2 (0.9)	60 (5.2)
pT2	14 (6.3)	189 (16.2)
pT3	159 (71.6)	740 (63.5)
pT4	47 (21.2)	176 (15.1)
pN stage		
pN0	124 (56.1)	587 (51.4)
pN1	50 (22.6)	308 (27.0)
pN2	47 (21.3)	247 (21.6)
Tumor grade		
G1	4 (1.8)	27 (2.3)
G2	167 (75.9)	1010 (86.7)
G3	49 (22.3)	128 (11.0)
Vascular invasion		
Absent	168 (76.7)	834 (71.5)
Present	51 (23.3)	332 (28.5)
Age (years)		
Mean	69	70
Tumor diameter (mm)		
Mean	59.5	47.5
Survival time (months)		
Median	142.0	73.0
5-year survival rate	71.2%	53.6%
10-year survival rate	62.4%	38.1%

Frequency of CK20, CK7 and CDX2 Expression in Colorectal Cancer Stratified by Mismatch Repair Status

The mean percentage of positive tumor cells expressing CK20 and CDX2 was higher in mismatch repair-proficient compared with mismatch repair-deficient colorectal cancer ($P < 0.001$) (Table 2). There was no statistically significant difference in CK7 expression between mismatch repair-proficient and mismatch repair-deficient colorectal cancer ($P = 0.743$). In addition, tumors were analyzed according to localization within the colorectum. Rectal cancers ($n = 482$) were significantly more frequently positive for CK20 ($P < 0.001$) and CDX2 ($P < 0.001$) but not for CK7 ($P = 0.987$).

Table 2 Mean percentage of positive tumor cells expressing CK20, CK7 and CDX2 in mismatch repair-proficient and -deficient colorectal cancer

Protein	Mean (%) $\pm$ s.d.		P-value
	Mismatch repair-proficient	Mismatch repair-deficient	
CK20	73.5 $\pm$ 36.6	58.9 $\pm$ 43.8	< 0.001
CK7	4.3 $\pm$ 18.5	5.3 $\pm$ 21.0	0.743
CDX2	84.3 $\pm$ 31.4	60.9 $\pm$ 41.9	< 0.001

Abbreviation: s.d., standard deviation.

Figure 1 Percentage of CDX2- and CK20-negative cases is higher in mismatch repair-deficient (19.3 and 21.6%) compared with mismatch repair-proficient colorectal cancer (6.7 and 7.5%).

CK20/7 Phenotypes in Mismatch Repair-Proficient and -Deficient Colorectal Cancer

In mismatch repair-deficient colorectal cancer CK20/7 phenotypes including CK20 negativity (CK20-/CK7- and CK20-/CK7+) were more frequent than in mismatch repair-proficient colorectal cancers (19.3% vs 7.5%) (Figure 1) (Table 3). The typical phenotype for colorectal cancer (CK20+/CK7-) was found in 84.5% of mismatch repair-proficient and in 73.1% of mismatch repair-deficient colorectal cancer.

CK20/7/CDX2 Phenotypes In Mismatch Repair-Proficient And Deficient Colorectal Cancer

In mismatch repair-deficient colorectal cancer CK20/7/CDX2 phenotypes including CDX2 negativity (CK20-/CK7-/CDX2-, CK20-/CK7+/CDX2-, CK20+/CK7-/CDX2- and CK20+/CK7+/CDX2-) could be more frequently detected compared with mismatch repair-proficient colorectal cancer (21.6% vs 6.7%) (Figure 1) (Table 4).

Table 3 Distribution of CK20 and CK7 expression in mismatch repair-proficient and -deficient colorectal cancer

Phenotype	Mismatch repair-deficient N (%)	Mismatch repair-proficient N (%)
CK20-/CK7-	33 (16.8)	64 (6.4)
CK20-/CK7+	5 (2.5)	11 (1.1)
CK20+/CK7-	144 (73.1)	845 (84.5)
CK20+/CK7+	15 (7.6)	80 (8.0)

Association of CK20, CK7 and CDX2 with Clinico-Pathological Parameters

Loss of CK20 was associated with higher tumor grade in mismatch repair-proficient and -deficient colorectal cancer ($P < 0.001$) and with location (right side) in mismatch repair-deficient colorectal cancer ($P = 0.02$). In mismatch repair-proficient colorectal cancer there was an association between loss of CDX2 and higher T stage ($P < 0.001$), N stage ($P < 0.001$), tumor grade ($P < 0.001$), vascular invasion ($P = 0.007$) and location (right side) ($P < 0.001$), whereas in mismatch repair-deficient colorectal cancer CDX2 loss was associated with location (right side) ($P = 0.013$) (Tables 5–7).

Multivariable Analysis

In mismatch repair-proficient colorectal cancer CK20 expression was an independent adverse prognostic factor adjusting for T and N, grade, vascular invasion, age and tumor side (left vs right) (P -value = 0.002; HR (95% CI) = 1.39 (1.14–1.69)), whereas CDX2 ($P = 0.958$) and CK7 ($P = 0.129$) were not independent prognostic factors.

Survival Analysis of CK20 Expression in Mismatch Repair-Proficient Colorectal Cancer Stratified by Intratumoral Lymphocytes

To further investigate the prognostic role of CK20 in mismatch repair-proficient colorectal cancer, the association between CK20 expression and intratumoral lymphocytes was analyzed. CK20 positivity was associated with absence of CD8+ intratumoral lymphocytes in both mismatch repair-deficient ($P < 0.001$) and mismatch repair-proficient ($P = 0.02$) tumors. Additionally, the stratification of the CK20 analysis by intratumoral lymphocytes showed a better survival in subgroups with presence of intratumoral lymphocytes independently of CK20 expression. Indeed, CK20-positive colorectal cancers with presence of intratumoral lymphocytes had a better survival than CK20-negative colorectal cancer without intratumoral lymphocytes ($P < 0.001$) (Figure 2).

Table 4 Distribution of CK20, CK7 and CDX2 expression in mismatch repair-proficient and -deficient colorectal cancer

Phenotype	Mismatch repair-deficient N (%)	Mismatch repair-proficient N (%)
CK20-/CK7-/CDX2-	18 (9.5)	19 (1.9)
CK20-/CK7+/CDX2-	3 (1.6)	7 (0.7)
CK20+/CK7-/CDX2-	20 (10.5)	33 (3.4)
CK20+/CK7+/CDX2-	0 (0.0)	7 (0.7)
CK20-/CK7-/CDX2+	13 (6.8)	42 (4.3)
CK20-/CK7+/CDX2+	2 (1.1)	4 (0.4)
CK20+/CK7-/CDX2+	119 (62.6)	795 (81.1)
CK20+/CK7+/CDX2+	15 (7.9)	73 (7.5)

Discussion

According to the revised Bethesda guidelines,³⁸ several pathologic features are associated with microsatellite instability status including the presence of intratumoral lymphocytes, a Crohn-like lymphocytic reaction, mucinous or signet ring differentiation, and a medullary or undifferentiated growth pattern.^{24–32} Jenkins *et al*³⁹ recently proposed a score to predict microsatellite instability high colorectal cancer based on characteristic features associated with this subgroup of tumors including age at diagnosis, anatomical site, histologic subtype, tumor grade, Crohn-like reaction and intratumoral lymphocytes. The immunohistochemical phenotype of microsatellite instability high colorectal cancer is poorly understood. Indeed, in bioptic material the ‘traditional’ multi-marker phenotype CK20/CK7/CDX2 is often applied to identify primary colorectal cancer or a colorectal cancer metastasis. However, its expression in colorectal cancer stratified by mismatch repair status has never previously been systematically analyzed.

In this study, a large series of colorectal cancers was stratified by immunohistochemistry into 1197 mismatch repair-proficient and 223 mismatch repair-deficient subgroups. Molecular testing was not performed on this cohort of patients as the sensitivity for determining microsatellite instability status by immunohistochemistry alone is reported to exceed 90% and the predictive value of absence of expression of either MLH1 or MSH2 for predicting microsatellite instability high has previously been described at 100%.⁴⁰ Additionally, microsatellite instability testing is recognized as infeasible in standard pathology laboratories and is currently not recommended in routine practice.⁴¹

One of the most obvious issues concerning the use of tissue microarrays is the extent of tumor heterogeneity, which may result from sampling of only one core of 0.6 mm in diameter. Bubendorf *et al* have reviewed this concern in depth.⁴² Several large studies in breast cancer, bladder carcinoma and with multiple tumor entities (multitumor array) have all reproduced findings from large whole tissue

Table 5 Association of CK20 with clinico-pathological features in mismatch repair-proficient and -deficient colorectal cancer

Feature (cutoff score)	Mismatch repair-proficient		P-value	Mismatch repair-deficient		P-value
	Negative N (%)	Positive N (%)		Negative N (%)	Positive N (%)	
<i>T stage (95%)</i>						
Early (T1+T1)	123 (55.4)	99 (44.6)	0.489	9 (60.0)	6 (40.0)	0.566
Late (T3+T4)	454 (58.1)	327 (41.9)		128 (68.8)	58 (31.2)	
<i>N stage (95%)</i>						
N0	295 (57.7)	216 (42.3)	0.897	82 (70.7)	34 (29.3)	0.338
>N0	276 (58.2)	198 (41.8)		54 (64.3)	30 (35.7)	
<i>Tumor grade (70%)</i>						
Early (G1+G2)	254 (27.8)	659 (72.2)	<0.001	62 (39.5)	95 (60.5)	<0.001
Late (G3)	42 (46.7)	48 (53.3)		29 (69.0)	13 (31.0)	
<i>Vascular invasion (85%)</i>						
Absence	248 (34.4)	472 (65.6)	0.09	75 (48.7)	79 (51.3)	0.494
Presence	114 (40.1)	170 (59.9)		24 (54.6)	20 (45.5)	
<i>Side (90%)</i>						
Left-sided	425 (58.3)	304 (41.7)	0.723	27 (39.1)	42 (60.9)	0.02
Right-sided	161 (56.9)	122 (43.1)		75 (56.4)	58 (43.6)	
Median S(t) (months) (85%)	93.0 (77.0-ne)	63.0 (54.0–75.0)	0.041 ^a	Not reached	Not reached	0.63
Mean ± s.e.	71.3 ± 2.5	65.8 ± 1.9		71.5 ± 3.5	80.9 ± 4.7	

^aIndependent adverse prognostic factor (adjusting for T and N, grade, vascular invasion, age and tumor side (left vs right) (P -value = 0.002) HR (95% CI) = 1.39 (1.14–1.69).

Table 6 Association of CK7 with clinico-pathological features in mismatch repair-proficient and -deficient colorectal cancer

Feature (cutoff score)	Mismatch repair-proficient		P-value	Mismatch repair-deficient		P-value
	Negative N (%)	Positive N (%)		Negative N (%)	Positive N (%)	
<i>T stage (5%)</i>						
Early (T1+T1)	217 (96.4)	8 (3.6)	0.147	14 (93.3)	1 (6.7)	1
Late (T3+T4)	761 (93.6)	52 (6.4)		180 (93.3)	13 (6.7)	
<i>N stage (5%)</i>						
N0	506 (94.8)	28 (5.2)	0.589	111 (93.3)	8 (6.7)	0.735
>N0	457 (93.8)	30 (6.2)		81 (42.2)	7 (8.0)	
<i>Tumor grade (5%)</i>						
Early (G1+G2)	888 (94.6)	51 (5.4)	0.489	149 (93.7)	10 (6.3)	0.34
Late (G3)	92 (92.9)	7 (12.1)		42 (89.4)	5 (10.6)	
<i>Vascular invasion (5%)</i>						
Absence	709 (95.0)	37 (5.0)	0.135	149 (94.3)	9 (5.7)	0.115
Presence	271 (92.5)	22 (7.5)		41 (87.2)	6 (12.8)	
<i>Side (5%)</i>						
Left-sided	705 (93.5)	49 (6.5)	0.244	68 (94.4)	4 (5.6)	0.51
Right-sided	284 (95.6)	13 (4.4)		126 (92.0)	11 (8.0)	
Median S(t) (months) (5%)	78.0 (65.0–88.0)	45.0 (30.0-ne)	0.332	Not reached	65.5 (11.0-ne)	0.032 ^a
Mean ± s.e.	68.9 ± 1.5	40.7 ± 2.9		83.2 ± 3.2	49.2 ± 10.1	

^aCK7 is not an independent prognostic factor in mismatch repair-deficient colorectal cancer (P = 0.129).

sections using tissue microarrays. They stress that the larger the number of tissues sampled, the less likely heterogeneity may play a role particularly as the likelihood of error in sampling will be similar in all tumor groups represented on one array. In

addition, it should be underlined that tissue microarrays are not used for individual case assessment, but are designed for high throughput population-based phenotypic and genotypic evaluation.⁴³ Therefore, associations between molecular

Table 7 Association of CDX2 with clinico-pathological features in mismatch repair-proficient and -deficient colorectal cancer

Feature (cutoff score)	Mismatch repair-proficient		P-value	Mismatch repair-deficient		P-value
	Negative N (%)	Positive N (%)		Negative N (%)	Positive N (%)	
<i>T stage (95%)</i>						
Early (T1+T2)	46 (20.3)	181 (79.7)	<0.001	7 (50.0)	7 (50.0)	0.252
Late (T3+T4)	275 (34.0)	535 (66.0)		125 (66.1)	64 (33.9)	
<i>N stage (95%)</i>						
N0	138 (25.8)	396 (74.2)	<0.001	75 (65.2)	40 (34.8)	0.9
>N0	180 (37.0)	307 (63.0)		56 (64.4)	31 (35.6)	
<i>Tumor grade (85%)</i>						
Early (G1+G2)	184 (19.6)	757 (80.5)	<0.001	83 (53.9)	71 (46.1)	0.085
Late (G3)	44 (45.8)	52 (54.2)		32 (68.1)	15 (31.9)	
<i>Vascular invasion (95%)</i>						
Absence	211 (28.3)	534 (71.7)	0.007	94 (61.8)	58 (38.2)	0.119
Presence	108 (36.9)	185 (63.1)		36 (75.0)	12 (25.0)	
<i>Side (100%)</i>						
Left-sided	208 (27.6)	546 (72.4)	<0.001	37 (27.8)	32 (46.4)	0.013
Right-sided	118 (39.9)	178 (60.1)		96 (71.1)	39 (28.9)	
Median S(t) (months) (90%)	54.0 (36.0–85.0)	81.0 (65.0–93.0)	0.007 ^a	Not reached	Not reached	0.197
Mean $\pm$ s.e.	61.7 $\pm$ 2.8	70.7 $\pm$ 1.7		77.5 $\pm$ 4.1	67.7 $\pm$ 3.6	

^aCDX2 is not an independent prognostic factor adjusting for T, N stage, tumor grade, vascular invasion and age ($P=0.958$).

Figure 2 Kaplan-Meier survival curve in mismatch repair-proficient colorectal cancer of (1) CK20– with presence of intratumoral lymphocytes, (2) CK20+ with presence of intratumoral lymphocytes, (3) CK20– with absence of intratumoral lymphocytes, (4) CK20+ with absence of intratumoral lymphocytes ($P<0.001$).

alterations and clinical and morphological parameters are likely to be similarly detectable on sufficiently large tissue microarrays as in large section analyses. Although it is possible that a small proportion of presumed mismatch repair-deficient colorectal cancer cases were incorrectly assigned, the overall findings are likely to be valid in view of the large numbers of samples and the good fit with clinico-pathological features.

Our results highlighted a significant difference in the mean percentage of CK20- and CDX2-positive

cells in mismatch repair-proficient and -deficient colorectal cancer ($P<0.001$). Additionally, multi-marker combinations with CDX2 and CK20 negativity were more frequently associated with mismatch repair-deficient (19.3 and 21.6%) compared with mismatch repair-proficient colorectal cancer (6.7 and 7.5%). The two most frequent marker combinations other than CK20+/CK7– such as CK20–/CK7– and CK20+/CK7+ were observed in 16.8 and 7.6%, respectively.

In daily practice, the phenotype CK20–/CK7– is used to clarify the diagnosis of hepatocellular carcinoma, renal cell carcinoma, prostate carcinoma, squamous cell and small cell lung cancer as well as head and neck carcinoma. Our findings suggest that microsatellite instability high colorectal cancer should be added to this list of differential diagnoses expressing this combination of markers.

In addition, CK20+/CK7+ is frequently attributed to urothelial tumors, ovarian mucinous adenocarcinoma, pancreatic adenocarcinoma and cholangiocarcinoma.⁵ Again, microsatellite instability high colorectal cancer cannot be excluded from the panel of possible diagnoses exhibiting this immunohistochemical phenotype.

CDX2 negativity was observed in 21.6% of microsatellite instability high colorectal cancer and is therefore not likely to narrow the differential diagnosis, which can be made by CK20 and CK7. Although CDX2 is known to be expressed in adenocarcinomas of intestinal origin^{18,44} and also in primary ovarian mucinous tumors,¹⁹ CDX2 expression has been described in tumors at various

anatomic sites.^{19,45–50} On the basis of this ubiquity, negative CDX2 expression in adenocarcinoma cannot systematically rule out a diagnosis of colorectal cancer. Especially, the immunohistochemical analysis of a mucinous adenocarcinoma with negative CK20 expression in addition to negative CDX2 expression, located on the right side of the colon of a female patient must be underlined as a potential pitfall of misdiagnosis, where a metastasis could be diagnosed without consideration of a possible microsatellite instability high colorectal cancer.

Our findings are in agreement with the results of a previous study where 32% of the microsatellite instability high colorectal cancers were CK20 negative compared with 9% of mismatch repair-proficient colorectal cancer.³³ CK20 negativity in colorectal cancer has been reported to range between 0 and 9%.^{4,11,51–54} Park *et al*¹¹ observed an association between CK20 loss, higher tumor grade and right-sided location and Hinoi *et al*⁵⁵ observed an association between loss of CDX2, microsatellite instability high status, right-sided tumor location and poor differentiation.

To study systematically the prognostic role of CK20, CK7 and CDX2 we additionally analyzed the associations of these markers with clinico-pathological findings including T stage, N stage, tumor grade, vascular invasion, tumor location and survival using ROC curve analysis. In mismatch repair-proficient colorectal cancer loss of CK20 was associated with higher tumor grade whereas loss of CDX2 showed an association with higher T stage, N stage, and tumor grade, with vascular invasion and right sidedness. In mismatch repair-deficient colorectal cancer there was an association between loss of CK20, higher tumor grade and right-sided location. CDX2 loss was only associated with right sidedness in this subgroup of tumors.

In both, mismatch repair-proficient and -deficient colorectal cancer subsets loss of CK20 was associated with higher tumor grade, whereas overexpression of this marker in mismatch repair-proficient colorectal cancer was found to be an independent adverse prognostic indicator. This apparent contradiction may be explained by several factors: first, higher tumor grade does not necessarily imply worse survival, as is underlined by the high percentage of microsatellite instability high colorectal cancer, which are poorly differentiated but have a relatively good prognosis compared with micro-satellite stable tumors; second, most cases of distant colorectal cancer metastases express CK20; third, we have shown in our study that loss of CK20 is associated with more abundant intratumoral lymphocytes, which could be linked to the observed more favorable prognosis; indeed CK20-negative cases with presence of intratumoral lymphocytes showed a better survival than CK20-positive colorectal cancers without intratumoral lymphocytes; fourth, using ROC curve analysis the cutoff for declaring a case CK20 'positive' was 85% in contrast

to the often used cutoff of 0% (mainly for diagnostic purposes).

The focus of the present study was to elucidate the differential diagnostic and prognostic role of CK20, CK7 and CDX2 in colorectal cancer stratified by mismatch repair status. Loss of CDX2 and CK20 overexpression are markers of tumor progression in mismatch repair-proficient colorectal cancer. Loss of CDX2 and CK20 are more frequently encountered in mismatch repair-deficient colorectal cancer, which should be taken into consideration to correctly differentiate between primary and metastatic colorectal cancer.

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