Expression of Cytokeratin 20 Indicates Invasive Histological Phenotype in Poorly Differentiated Colorectal Adenocarcinoma

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Abstract. *Background/Aim: Cytokeratin (CK) 20 expression is an independent prognostic factor of poorly differentiated adenocarcinoma (PDA) of the colon and rectum. We aimed to investigate the mechanism of its involvement through a clinicopathological study. Patients and Methods: We analyzed 156 surgically resected PDAs, which were sub-classified as solid type (Por1) showing expansive growth, or non-solid type (Por2) showing infiltrative growth. Associations of CK20 expression with morphological features and molecular markers were analyzed. Results: CK20$^+$ PDA (n=91) was associated with more advanced disease stage and unfavorable prognosis compared with CK20$^-$ PDA (n=65). Pathologically, CK20$^+$ PDA was significantly associated with p53 overexpression, Por2, abundant fibrous stroma, and stepwise de-differentiation, while CK20$^-$ PDA was significantly associated with mismatch repair deficiency, Por1, sparse fibrous stroma, and de novo histogenesis. Conclusion: CK20 expression in PDA is closely associated with invasive histological features, providing prognostic significance, and may also point to a specific histogenetic pathway.*

To date, more than 20 subtypes of cytokeratin (CK) have been classified and numbered based on molecular weight and isoelectric pH (1, 2). CK7 is expressed in numerous ductal and glandular epithelia, including those of lung, breast, ovary, and endometrium, while CK20 is expressed in the gastrointestinal epithelium, urothelium and in Merkel cells. Coordinate CK7/CK20 expression profiles have been extensively studied in a variety of primary and metastatic carcinomas (3-8). The CK7$^-$/CK20$^+$ profile is known to be highly characteristic of colorectal cancer (CRC); however, not all CRCs exhibit this profile. A substantial proportion of CRC was reported to be either CK7$^+$ or CK20$^-$, particularly in right-sided and high-grade CRCs, consisting of poorly differentiated adenocarcinoma (PDA) and undifferentiated carcinoma, among others (9). CRC may be classified into adenocarcinoma, mucinous adenocarcinoma, signet-ring cell carcinoma, squamous cell carcinoma, adenosquamous carcinoma, medullary carcinoma, undifferentiated carcinoma, in addition to other rare variants described in the World Health Organization (WHO) classification of colorectal cancer (10). Previously, we investigated CK7/CK20 profiles of various histological CRC subtypes. Normal colonic mucosa usually expresses CK20, and we expected that loss of CK20 might suggest marked de-differentiation and disease progression, and accordingly, an unfavorable prognosis. CK20 expression was retained in 61 out of 63 (96.8%) well- and moderately differentiated adenocarcinomas (WMDAs) and 47 out of 91 (51.7%) PDAs with less than 50% glandular structural components (11). Surprisingly, survival analysis revealed that loss of CK20 expression was associated with significantly favorable prognosis in PDA (11). In the present study, we performed immunohistochemical analyses of mismatch repair (MMR) deficiency and p53 overexpression. In addition, we histologically-classified PDA as solid type (Por1) or non-solid type (Por2), semi-quantified amounts of fibrous stroma, and searched for histological evidence of disease progression from WMDA to PDA. We also investigated associations between these histological features and CK20 expression status to explore the mechanism of an unfavorable prognosis of PDA with CK20 expression.

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Key Words: Poorly differentiated adenocarcinoma, colon, cytokeratin 20, solid type (Por1), non-solid type (Por2).

Patients and Methods

Patients and tumor samples. PDAs were selected from all CRCs surgically resected at the Dokkyo Medical University Koshigaya Hospital (DMUKH) between 1990 and 2011 (tumors 1-53), Tokyo Kosei Nenkin Hospital (TKNH) between 1991 and 2010 (tumors

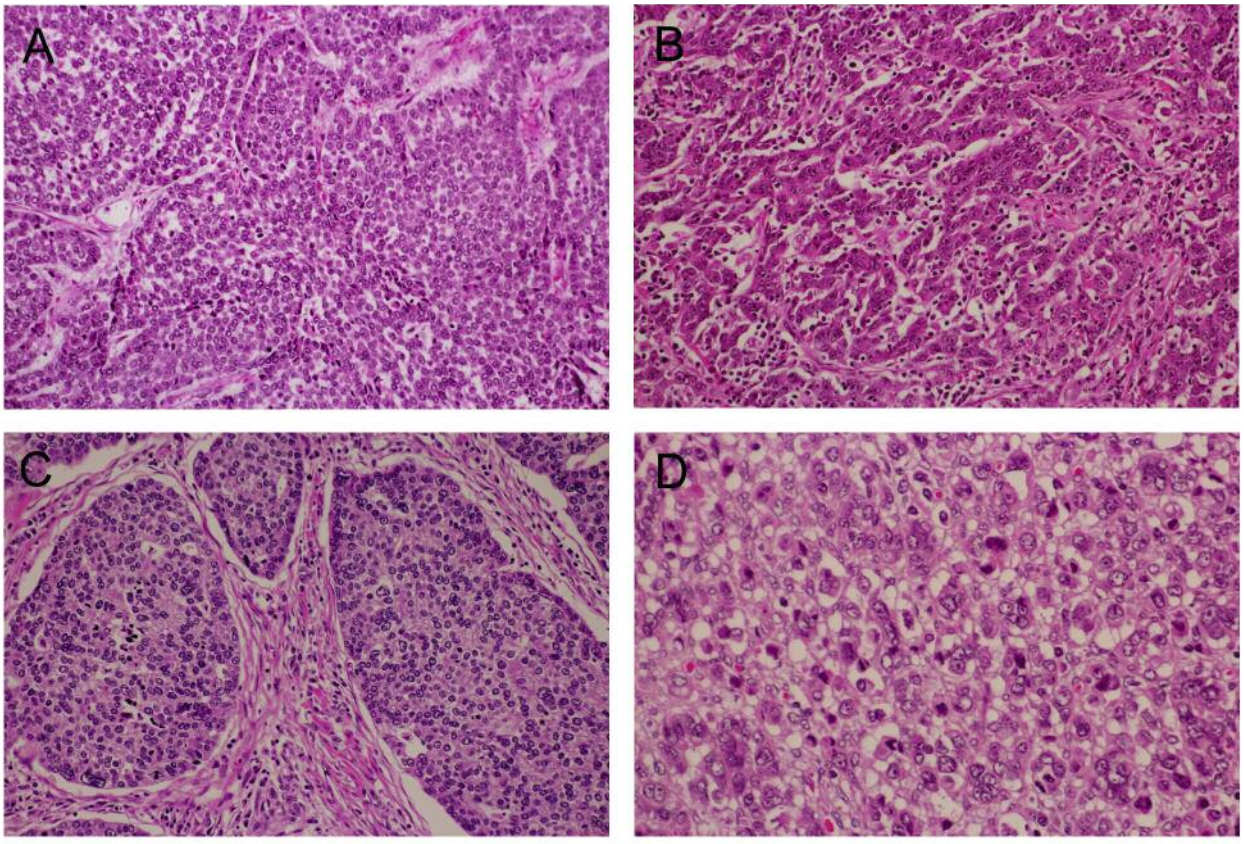

Figure 1. *Sub-classification of poorly differentiated adenocarcinoma (PDA). A: Solid type (Por1) tumor. Tumor 144, showing pushing margin with med fibrous stroma [hematoxylin and eosin (H&E) staining, ×20]. B: Non-solid type (Por2) tumor. Tumor 154, showing infiltrative margin with int fibrous stroma (H&E staining, ×20). C: Por1 with int fibrous stroma. Tumor 82 (H&E staining, ×20). D: Por2 with med fibrous stroma. Tumor 140 (H&E staining, ×40).*

54-90), Saiseikai Kawaguchi General Hospital (SKGH) between 2000 and 2011 (tumors 91-141), and the International University of Health and Welfare Shioya Hospital (IUHWSH) between 2000 and 2011 (tumors 142-156) solely on the basis of tumor histology, and included 155 patients with a total of 156 resected tumors. Solid-type PDAs (tumors 67 and 68) were derived from one patient with hereditary non-polyposis colorectal cancer. For comparison, WMDAs surgically-resected at TKNH between April 1998 and March 2000 were also included, and consisted of 63 tumors from 63 patients (tumors 201-263). All tumors in this study were previously diagnosed as primary CRC.

Archival tissue blocks were obtained from the pathological departments of the involved hospitals. For survival analysis, patients with complete medical records were included and followed-up for five years, while individuals with invasive cancer originating from other sites were excluded. Clinicopathological classifications and stage groupings were performed based on the WHO classification of colorectal cancer and the tumor, node, metastases (TNM) staging system by the American Joint Committee on Cancer (10, 12). PDA was sub-classified as either Por1 or Por2. Por1 consisted of tumor cells with solid growth, typically accompanied by sparsely distributed stromal cells (case no. 144: Figure 1A). Tumor nests revealed mostly pushing margins. In contrast, Por2 consisted of tumor cells forming a trabecular structure or a small cluster of tumor cells, and was typically rich in fibrous stroma (case no. 154: Figure 1B). Tumor nests displayed an infiltrative margin. In cases exhibiting intermingling of Por1 and Por2 subtypes, classification was based on the predominant type. In cases displaying features of both Por1 and Por2 subtypes (for example, solid growth with abundant stroma (case no. 82: Figure 1C) or a trabecular/small clustering structure with sparse stroma (case no. 140: Figure 1D), classification was performed on the basis of cell growth pattern. The quantity of fibrous stroma was semi-quantified as either *med* (sparse fibrous stroma), *int* (intermediate between *med* and *sci*), or *sci* (abundant fibrous stroma). These classifications are consistent with those proposed by the Japanese Classification of Colorectal Carcinoma with modifications (13).

Histogenetic pattern was analyzed by investigating the boundary between non-neoplastic mucosa and cancer. PDA was then subclassified as either *de novo* type (*de novo* PDA) when PDA appeared abruptly next to the non-neoplastic mucosa (case no. 144 and 154: Figure 2A and B) or sequentially dedifferentiated type (PDA in the WMDA-PDA sequence or in the stepwise dedifferentiation) when sequential disease progression from non-neoplastic mucosa to adenoma and WMDA and from WMDA to PDA was observed (case no. 149 and 129: Figure 2C and D). This study protocol was approved by the ethical review boards of the participating hospitals: DMUKH, Koshigaya 23008; TKNH, 30/11/2011; SKGH, no. 24-5: IUHWSH, FK-94.

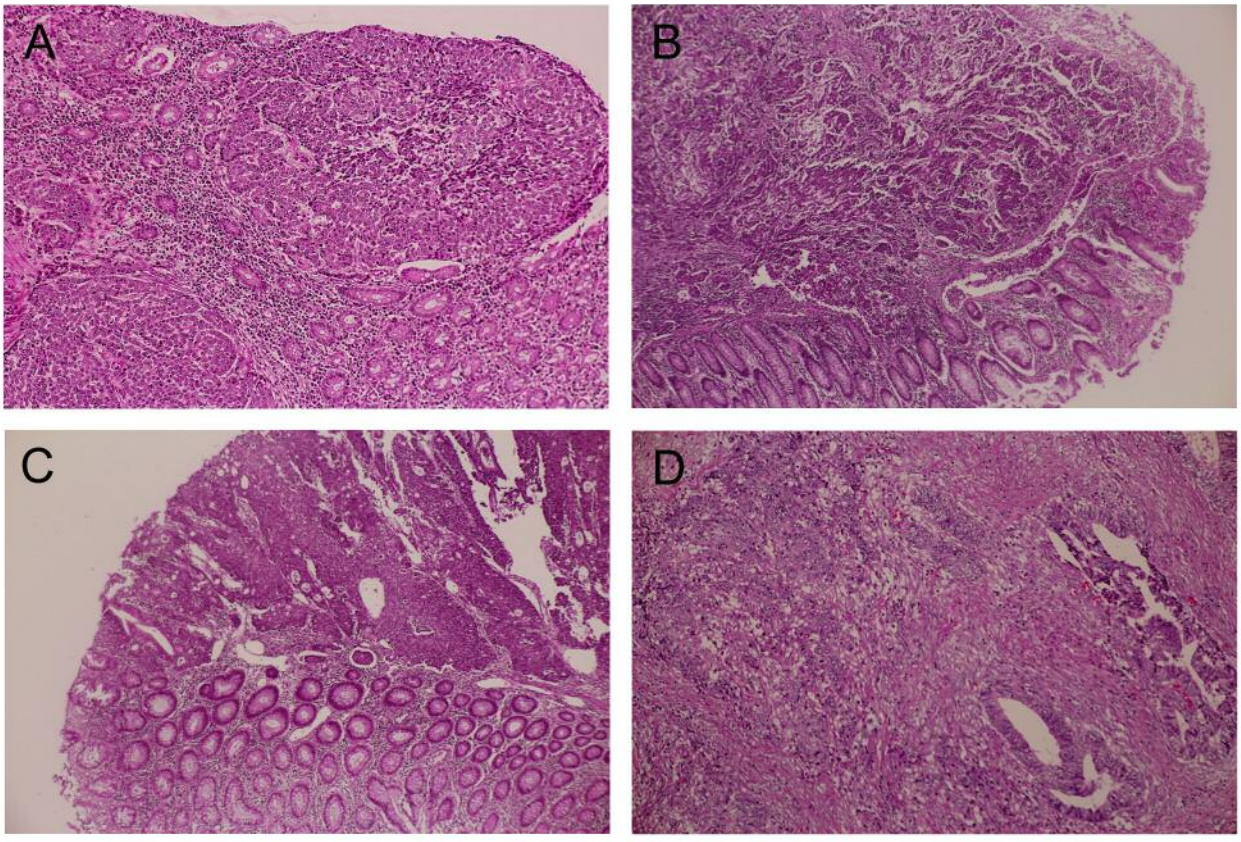

Figure 2. *Histogenetic analysis by investigating the boundary between non-neoplastic mucosa and cancer. A: De novo histogenesis of solid type (Por1) tumors. Tumor 144 [hematoxylin and eosin (H&E) staining, ×4]. B: De novo histogenesis of non-solid type (Por2) tumors. Tumor 154 (H&E staining, ×4). C: Stepwise dedifferentiation of Por1. Tumor 149 (H&E staining, ×4). D: Stepwise dedifferentiation of Por2. Tumor 129 (H&E staining, ×10).*

Immunohistochemistry. Tumor specimens were fixed in 10% neutral-buffered formalin for 48 h, embedded in paraffin, and cut into 4-μm-thick sections. Representative tissue blocks with the least differentiated carcinoma component were used.

For analysis of CK expression, antigen retrieval was performed using microwave irradiation for 5 min. Sections were incubated with primary antibodies detecting CK7 (mouse monoclonal, OV-TC, 1:100; Dako, Glostrup, Denmark) and CK20 (mouse monoclonal, Ks20.8, 1:100; Dako), for 30 min at room temperature. For analysis of human mutL homolog 1 (hMLH1), human mutS homolog 2 (hMSH2), and p53 overexpression, antigen retrieval was performed using microwave irradiation for 10 min, autoclaving (121˚C) for 5 min, and by heating at 95˚C for 20 min in a water bath, respectively. Primary antibodies detecting MLH1 (rabbit monoclonal, EPR3894, 1:400; GeneTex, San Antonio, TX, USA), MSH2 (rabbit polyclonal, 15520-1-AP, 1:200; Proteintec, Chicago, IL, USA), and p53 (mouse monoclonal, clone DO-7, 1:200; Dako) were used. Samples were treated overnight with primary antibodies at 4˚C. Immunostaining was performed using an N-Histofine Simple Stain MAX-PO kit (Nichirei, Tokyo, Japan). On the basis of expression levels in normal colonic mucosa, positive staining of ≥25% of tumor cells for CK20 and ≥1% of tumor cells for CK7 was evaluated as positive, respectively. The immunostaining results for MMR proteins were either completely negative (negative) or nearly 100% positive (positive). Tumors that failed to express either of the two proteins were considered MMR-deficient. For p53, strong positive staining of ≥25% of tumor cell nuclei was evaluated as positive for overexpression, which significantly matched the results of *p53* mutational analysis in the WMDA cases (data not shown).

Statistics. Specific parameters between two patient cohorts were compared using the chi-square test with or without Yates' correction, or the Fisher's exact test. Age was compared using the Mann–Whitney *U*-test, and survival curves were analyzed using Kaplan–Meier and log-rank tests. Univariate analyses were performed using Cox regression analysis for investigating associations between parameters and prognosis. Multivariate analyses were performed using a step wise variable elimination method with an entry limit of $p<0.10$ and a removal limit of $p>0.05$. A value of $p<0.05$ was considered significant. Statistical analyses were performed using IBM SPSS Statistics 20 (IBM, Armonk, NY, USA).

Results

Clinicopathological characteristics of PDA and comparison with WMDA. Clinicopathological characteristics of PDA were analyzed and compared to those of WMDA. A total of 156

Table I. *Clinicopathological characteristics of well- and moderately differentiated adenocarcinoma (WMDA) and poorly differentiated adenocarcinoma (PDA).*

	WMDA (n=63)	PDA (n=156)			*p*-Value			
		Total (n=156)	CK20$^-$ (n=65)	CK20$^+$ (n=91)	WMDA *vs.* total PDA	CK20$^-$ PDA *vs.* CK20$^+$ PDA	WMDA *vs.* CK20$^-$ PDA	WMDA *vs.* CK20$^+$ PDA
Median age	65	66	70	65	0.464	0.176	0.142	0.993
Range	32-87	27-92	35-92	27-92				
Gender								
Male	39	79	29	50	0.086	0.203	0.033	0.468
Female	24	77	36	41				
Family history of CRC								
Yes	5	5	3	2	0.266	0.649	1.000	0.273
No	58	107	45	62				
Unknown		44	17	27				
Location								
Proximal	20	86	47	39	0.002	0.000	0.000	0.163
Distal	43	70	18	52				
Depth								
Up tp MP	10	6	2	4	0.004	0.686	0.031	0.031
Beyond MP	53	149	62	87				
Unknown		1	1					
Venous invasion								
Yes	48	133	52	81	0.086	0.173	0.486	0.034
No	15	22	12	10				
Unknown		1	1					
Lymphatic invasion								
Yes	35	143	56	87	0.000	0.061	0.000	0.000
No	28	12	8	4				
Unknown		1	1					
Nodal metastasis								
Yes	34	114	39	75	0.003	0.003	0.367	0.000
No	29	39	24	15				
Unknown		3	2	1				
Chemotherapy								
Yes	17	78	25	53	0.000	0.023	0.063	0.000
No	46	65	33	32				
Unknown		13	7	6				
Irradiation								
Yes	4	5	1	4	0.281	0.324	0.367	0.723
No	59	138	57	81				
Unknown		13	7	6				
TNM stage								
I/II	29	32	20	12	0.000	0.006	0.087	0.000
III/IV	34	123	44	79				
Unknown		1	1					
MMR deficiency								
Yes	1	35	29	6	0.000	0.000	0.000	0.250
No	56	121	36	85				
Unknown	6							
p53 overexpression								
Yes	31	90	28	62	0.303	0.002	0.434	0.024
No	31	66	37	29				
Unknown	1							

CRC, Colorectal cancer; CK, cytokeratin; TNM, tumor, node, metastases; MP, *muscularis propria*; MMR, mismatch repair.

PDA tumors and 63 control WMDAs were analyzed. PDA tended to be located in the proximal colon, and tumor stage of progression in terms of depth of invasion, lymphatic invasion, nodal metastasis, and TNM stage, was significantly more advanced than that of WMDA. In accordance with these results, patients with PDA were significantly more likely to undergo chemotherapy compared to patients with WMDA. PDA was slightly more frequent in female than in male

patients compared with WMDA. MMR deficiency was significantly more frequent in PDA than in WMDA. These characteristics of PDA are in accordance with those of previous studies (14-16) (Table I).

Clinicopathological characteristics of PDA in association with CK20 expression status. We previously reported that CK20 expression is a significant prognostic predictor of PDA (11). In this study, we combined the first and the second PDA cohorts in our previous study and analyzed them as a single cohort. Of the 156 PDAs, 50 (32.1%) were positive and 106 (67.9%) were negative for CK7, and 91 (58.3%) were positive and 65 (41.7%) were negative for CK20. No significant differences in age, gender, family history of CRC, depth of tumor invasion, venous invasion, lymphatic invasion, and irradiation were observed between $CK20^+$ and $CK20^-$ PDAs. In contrast, we observed a significant difference in tumor location, nodal metastasis, chemotherapy, and TNM stage between $CK20^+$ and $CK20^-$ PDAs. $CK20^-$ PDA was observed in the proximal colon significantly more often than in the distal colon. Nodal metastasis was significantly more frequent in $CK20^+$ PDA than in $CK20^-$ PDA. Patients with $CK20^+$ PDA underwent chemotherapy significantly more frequently than patients with $CK20^-$ PDA. $CK20^+$ PDAs exhibited more advanced TNM stage compared with $CK20^-$ PDAs. MMR deficiency and p53 overexpression were observed in 35 (22.4%) and 90 (57.7%) PDAs, respectively. MMR deficiency was significantly more frequent in $CK20^-$ PDA, while p53 overexpression was significantly more frequent in $CK20^+$ PDA. Similar trends in patients' gender, tumor location, and MMR proficiency were observed in $CK20^+$ PDA and WMDA. In contrast, depth of tumor invasion, venous and lymphatic invasion, nodal metastasis, chemotherapy, and p53 overexpression were significantly more frequent in $CK20^+$ PDA than in WMDA. This is in contrast with comparison between $CK20^-$ PDA and WMDA. These results are summarized in Table I.

Cox regression analysis revealed that CK20 expression and TNM stage were the only significant prognostic predictors of disease-free survival and overall survival (Table II). The disease-free survival was significantly higher in $CK20^-$ PDA than in $CK20^+$ PDA, and comparable to that of WMDA. Overall survival was also significantly higher in $CK20^-$ PDA compared with $CK20^+$ PDA, although inferior to that of WMDA (Figure 3).

Histological sub-classification and histogenetic analysis of PDA. PDA was sub-classified according to the histological structure of tumor cell clusters. PDA comprised of 62 Por1 and 94 Por2 cases, with a stromal tissue component of 23 *med*, 106 *int*, and 27 *sci*. The stromal tissue component of Por1 was *med* in 22 and *int* in 40, while those of Por2 were *med* in one, *int* in 66, and *sci* in 27. The amount of stromal tissue in Por1 was significantly greater than that in Por2. As

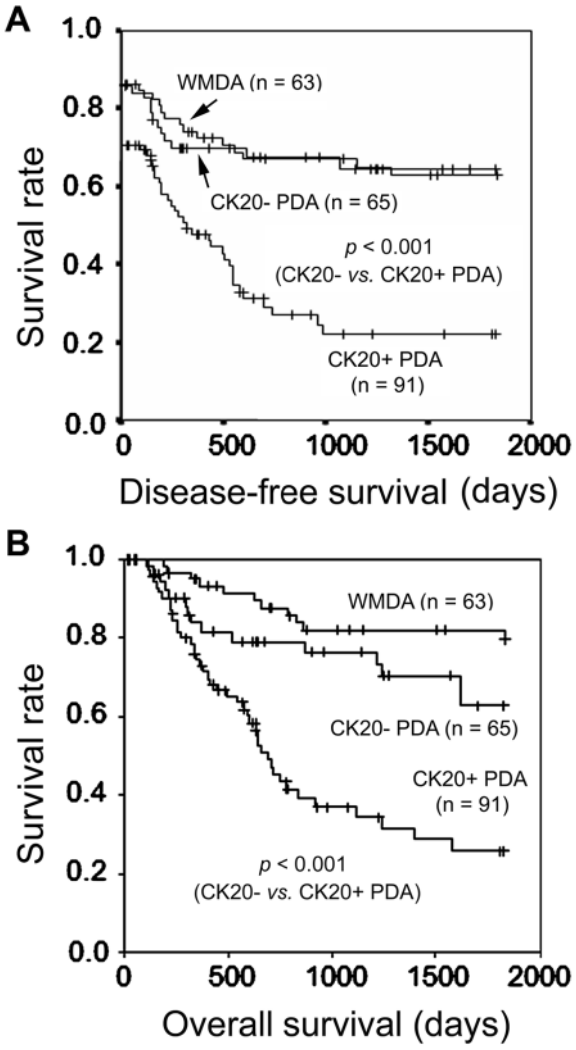

Figure 3. *The disease-free (A) and overall (B) survival curves of well- and moderately differentiated adenocarcinoma (WMDA), cytokeratin (CK) 20$^-$ and CK20$^+$ poorly differentiated adenocarcinoma (PDA) shown by the Kaplan-Meier method.*

for histogenesis, Por1 consisted of 34 *de novo* cancers and 28 cancers in stepwise de-differentiation, while Por2 consisted of 29 *de novo* cancers and 65 cancers in stepwise dedifferentiation. Thus, we observed a significant tendency in Por1 to favor *de novo* occurrence and Por2 to favor stepwise de-differentiation (Table III).

Histological sub-classification of PDA in association with CK20 expression status. We next investigated the associations between CK20 status and histological subclassification, MMR deficiency, and p53 overexpression in PDA. $CK20^-$ PDA consisted of 35 Por1 and 30 Por2, with a stromal tissue component of 17 *med*, 40 *int*, and 8 *sci*. $CK20^+$ PDA consisted

Table II. *Cox regression analysis of prognostic parameters in poorly differentiated adenocarcinoma (PDA).*

		Total PDA (n=156)	
	Parameter	HR (95% CI)	*p*-Value
Disease-free survival	Univariate analysis		
	Age (≥65)	1.007 (0.533-1.904)	0.982
	Gender (male)	0.692 (0.371-1.290)	0.246
	Location (proximal)	0.907 (0.489-1.682)	0.756
	TNM Stage (III/IV)	3.135 (1.316-7.469)	0.010
	Histology (Por1)	1.778 (0.917-3.446)	0.088
	Chemo-/radiotherapy (Yes)	2.194 (1.228-3.919)	0.008
	CK7-positive	0.784 (0.393-1.565)	0.490
	CK20-positive	2.711 (1.597-4.602)	0.000
	p53 overexpression (Yes)	1.443 (0.774-2.688)	0.248
	MMR-deficient	0.355 (0.157-0.803)	0.013
	Multivariate analysis		
	Chemo-/radiotherapy (Yes)	1.564 (0.982-2.492)	0.060
	TNM Stage (III/IV)	3.117 (1.412-6.881)	0.005
	CK20 positive	2.253 (1.322-3.839)	0.003
Overall survival	Univariate analysis		
	Age (≥65)	0.566 (0.338-0.949)	0.031
	Gender (male)	0.932 (0.555-1.566)	0.791
	Location (proximal)	0.696 (0.416-1.166)	0.169
	TNM Stage (III/IV)	2.734 (1.291-5.790)	0.009
	Histology (Por1)	1.122 (0.663-1.901)	0.668
	Chemo-/radiotherapy (Yes)	1.199 (0.721-1.994)	0.485
	CK7-positive	1.032 (0.595-1.790)	0.910
	CK20-positive	2.824 (1.556-5.126)	0.001
	p53 overexpression (Yes)	1.690 (0.992-2.878)	0.053
	MMR-deficient	0.552 (0.296-1.029)	0.061
	Multivariate analysis		
	TNM Stage (III/IV)	2.219 (1.036-4.755)	0.040
	CK20 positive	2.468 (1.348-4.518)	0.003

HR, Hazard ratio; CI, confidence interval; TNM, tumor node metastases; Por1, solid type; CK, cytokeratin; MMR, mismatch repair.

Table III. *Histological sub-classification and histogenetic analysis of poorly differentiated adenocarcinoma (PDA).*

	PDA (n=156)		
	Por1 (n=62)	Por2 (n=94)	*p*-Value
Amount of stromal tissue			
med	22	1	0.000
int	40	66	
sci	0	27	
Histogenesis			
De novo	34	29	0.003
Stepwise dedifferentiation	28	65	

Por1, Solid type; Por2, non-solid type.

Table IV. *Histological sub-classification of poorly differentiated adenocarcinoma (PDA) in association with cytokeratin (CK) 20 expression status.*

	PDA (n=156)		
	$CK20^-$ (n=65)	$CK20^+$ (n=91)	*p*-Value
Histological subclassification			
Por1	35	27	0.002
Por2	30	64	
Amount of stromal tissue			
med	17	6	0.002
int	40	66	
sci	8	19	
Histogenesis			
De novo	39	24	0.000
Stepwise dedifferentiation	26	67	

Por1, Solid type; Por2, non-solid type.

of 27 Por1 and 64 Por2 with a stromal tissue component of 6 *med*, 66 *int*, and 19 *sci*. These results suggest that $CK20^-$ PDA is associated with a higher percentage of Por1 and a smaller amount of stromal tissue component compared with $CK20^+$ PDA. As for histogenesis, $CK20^-$ PDA consisted of 39 *de novo* cancers and 26 cancers in stepwise de-differentiation, while $CK20^+$ PDA consisted of 24 *de novo* cancers and 67 cancers in stepwise de-differentiation. Thus, $CK20^-$ PDA was significantly associated with *de novo* occurrence while $CK20^+$ PDA was significantly associated with stepwise de-differentiation.

In summary, $CK20^-$ PDA significantly favored Por1, *de novo* histogenesis, and a smaller amount of stromal tissue. Conversely, $CK20^+$ PDA significantly favored Por2, histogenesis in stepwise de-differentiation, and a higher levels of stromal tissue. These results are summarized in Table IV.

Discussion

While CK20 expression is characteristic of CRC, not all cases of CRC express CK20. We recently reported that nearly half of PDAs do not express CK20 and that CK20 expression status can be a marker of patient prognosis in terms of disease-free and overall survival (11). This was initially observed following the analysis of 91 PDAs (the first cohort) and subsequently validated by analyzing 66 PDAs (the second cohort). We proposed several mechanisms, such as promoter methylation of transcription factors regulating the *CK20* gene associated with MMR deficiency and CpG island methylation phenotype (CIMP), and CK20-mediated cell motility. In this study, we further explored this mechanism by performing detailed histological analyses in a large patient cohort of 156 PDAs. One PDA was excluded from the present study because of unavailability of further material.

Compared to WMDA, PDA was significantly associated with proximal tumor location, more advanced depth of tumor invasion, lymphatic invasion, nodal metastasis, high TNM stage, performance of chemotherapy, and MMR deficiency, highlighting the possible association with high levels of microsatellite instability (MSI-H). Comparative analysis between $CK20^-$ and $CK20^+$ PDAs revealed that $CK20^-$ PDA was significantly associated with proximal tumor location, absence of nodal metastasis, low TNM stage, absence of chemotherapy, MMR deficiency, and absence of p53 overexpression, suggesting a possible association with MSI-H (14-18). Conversely, $CK20^+$ PDA was significantly associated with distal tumor location, nodal metastasis, high TNM stage, performance of chemotherapy, MMR proficiency, and p53 overexpression. It is well-known that mutation of the *p53* gene plays a critical role in the malignant transformation of CRC in the adenoma–carcinoma sequence of the multistep carcinogenetic model of CRC (19). *p53* gene mutations and p53 protein overexpression have been reported to be inversely associated with MSI-H (16, 18). This suggests that the majority of $CK20^+$ PDA may arise through pathogenetic pathways distinct from MMR deficiency. In fact, $CK20^+$ PDA revealed similarities with WMDA in terms of patient gender, tumor location, and MMR proficiency in contrast to $CK20^-$ PDA. However, depth of tumor invasion, venous and lymphatic invasion, nodal metastasis, chemotherapy, and p53 overexpression were significantly more frequent in $CK20^+$ PDA than in WMDA in contrast to comparison between $CK20^-$ PDA and WMDA. These data suggest that $CK20^+$ PDA may actually represent a more progressed form of WMDA.

The histopathological features of PDA in association with CK20 expression status were also investigated in detail. PDAs were sub-classified into Por1 and Por2, and the amount of fibrous stroma was also classified into *med*, *int* and *sci*, as proposed by the Japanese Society for Cancer of the Colon and Rectum (13). In this study, histological diagnosis of PDA was performed based on the least differentiated component of cancer according to the WHO classification (10). However, histological sub-classification of intermingling Por1 and Por2 cases was performed according to the most predominant feature, as proposed by the Japanese classification, because Por1 and Por2 cannot be classified on the basis of differentiation. The $CK20^-$ phenotype was significantly associated with Por1, *de novo* histogenesis, less fibrous stroma, and MMR deficiency, while the $CK20^+$ phenotype was significantly associated with Por2, stepwise de-differentiation, more abundant fibrous stroma, and p53 overexpression. These results suggest that many $CK20^-$ PDAs may arise as *de novo* cancer through mechanisms related to MMR deficiency or CIMP, while $CK20^+$ PDAs may arise *via* the stepwise de-differentiation pathway from WMDA to PDA in multistep carcinogenesis. Therefore, the majority of $CK20^+$ PDAs may not completely lose CK20 expression and bear *p53* mutations. It is well-known that *p53* mutations are a marker of unfavorable prognosis (20-22).

Generally, Por1 tumors grow expansively with a pushing margin and Por2 tumors grow infiltratively with a jagged margin, suggesting that Por2 might be more invasive than Por1. Indeed, Komori *et al*. investigated 78 cases of colorectal PDA, consisting of 29 Por1 and 49 Por2, and found that frequencies of nodal and organ metastases in the Por2 group were significantly higher than those in the Por1 group (23). The survival rate of the Por2 group was lower than that of the Por1 group, although this was not statistically significant. They concluded that prognosis of patients with Por2 tended to be unfavorable compared with these with Por1. In accordance with this study, we observed that Por2 demonstrated worse prognosis than Por1, although this was not statistically significant (data not shown). Our present data indicate that CK20 expression status is clearly a more powerful prognostic predictor of PDA than histological subclassification into Por1 or Por2. Growth of fibrous stroma around tumor cells, termed desmoplastic reaction, is the result of tumor cell infiltration and the amount of fibrous stroma is closely associated with the mode of invasion. Thus, it is reasonable that Por1 is associated with sparse stroma and Por2 with abundant stroma. Accordingly, we speculate that $CK20^+$ PDA may appear in the late stages of the stepwise de-differentiation pathway with a highly invasive growth pattern. Conversely, $CK20^-$ PDA may arise *de novo* with a malignant phenotype that is less aggressive than $CK20^+$ PDA.

In our previous study, we discussed whether down-regulation of CK20 may result from down-regulation of caudal type homeobox 1 (CDX1), which plays a role in intestinal epithelial cell differentiation (24). CDX1 is down-regulated by promoter hypermethylation in a number of CRC-derived cell lines, as well as in patient samples (25-28). CDX1 overexpression led to induction of CK20, while *CDX1*-knockdown resulted in down-regulation of CK20 in colon cancer cell lines. Promoter methylation is closely associated with CIMP, which underlies but does not completely coincide with MSI-H in CRC (29, 30). $CIMP^+$ CRCs exhibit characteristic clinical and pathological features that include origin in the proximal colon, higher frequency in females and poorly differentiated, mucinous histology (29, 31, 32). We therefore speculated that CIMP may be the underlying mechanism, leading to down-regulation of CDX1, resulting in decreased CK20 expression. Methylation of several CpG islands in the normal colonic mucosa has been shown to increase with age (33-35). Age-related methylation and subsequent inactivation of certain genes, such as *p16* (*INK4*) and *hMLH1*, has been suggested as a predisposing factor for increased risk of cancer with age (36, 37), and this may partly account for *de novo* carcinogenesis of Por1 tumors with MMR deficiency from normal colonic mucosa.

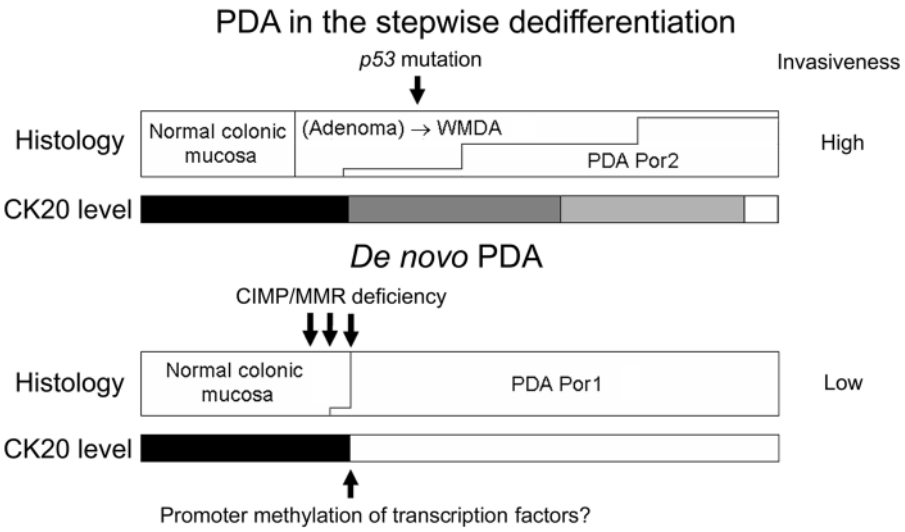

Figure 4. *Scheme of the two histogenetic pathways of poorly differentiated adenocarcinoma (PDA). Densities of cytokeratin (CK) 20 bars represent CK20 expression levels from positive, indicated by solid bars, to negative, indicated by open bars. WMDA, well- and moderately differentiated adenocarcinoma. Por1, solid type. Por2, non-solid type. CIMP, CpG island methylation phenotype. MMR, Mismatch repair.*

Why preservation of CK20 expression is associated with a more invasive PDA phenotype remains unknown. In our previous study, we discussed whether CK20 expression might be directly associated with metastasizing potential. This is based on the study showing that metastasizing CRCs typically express CK20 and previous reports have shown that CK20 expression regulated by peroxisome proliferator-activated receptor-γ enhanced invasiveness of tamoxifen-resistant MCF-7 breast cancer cells (3, 5, 6, 9, 38). In the present study, Por1 and Por2 comprised 53.8% (35/65) and 46.2% (30/65) of $CK20^-$ PDAs, respectively, and 29.7% (27/91) and 70.3% (64/91) of 91 $CK20^+$ PDAs, respectively. These data indicate that approximately half of $CK20^-$ PDAs grow infiltratively and that about a third of $CK20^+$ PDAs grow expansively. Therefore, we infer that down-regulation of certain cell adhesion molecules or up-regulation of cell motility-associated molecules may occur more frequently in Por2 tumors as a result of accumulated gene mutations during multistep carcinogenesis. Our hypothesis representing two distinct histogenetic pathways of $CK20^-$ and $CK20^+$ PDAs in the most typical form is summarized in Figure 4.

In conclusion, we performed a detailed investigation of the histological phenotype of $CK20^-$ and $CK20^+$ PDAs to gain deeper insight into CK20 expression and prognosis. $CK20^+$ PDA was more closely associated with a highly invasive histological phenotype and likely to occur following multistep de-differentiation, while $CK20^-$ PDA was more closely associated with a less invasive phenotype and likely to occur *de novo*. CK20 expression may be a marker of invasive PDA phenotype and may serve as a useful prognostic marker.

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