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Cytokeratin profiles identify diagnostic signatures in colorectal cancer using multiplex analysis of tissue microarrays

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Abstract. *Background and aims:* Recent cDNA expression profiling analyses indicate that within specific organ cancers Cytokeratins (CKs) dysregulation may identify subgroups with distinct biological phenotypes. Our objectives in this study were (1) to test whether cytokeratins were also distinct on the protein level, (2) to evaluate these biomarkers in a series of well-characterised CRCs, (3) to apply hierarchical cluster analysis to immunohistochemical data. *Methods:* Tissue microarrays (TMA) comprising 468 CRC specimens from 203 patients were constructed to evaluate *CK5*, *CK7*, *CK8*, *CK13*, *CK14*, *CK16*, *CK17*, *CK18*, *CK19* and *CK20*. In total, 2919 samples were analyzed. *Results:* Unsupervised hierarchical clustering discovered subgroups represented by reduced *CK8* and *CK20* expression, that differed by a shorter patients survival. The evaluation of the specific biomarkers by Kaplan–Meier analysis showed that reduced *CK8* expression ($p < 0.01$) was significantly associated with shorter patients' survival, but was not an independent factor correlated with tumour stage (pT), grading (G) and nodal stage (pN). *Conclusions:* Reduced coexpression of *CK8* and *CK20* may indicate an epithelial-mesenchymal transition (EMT) representing an important step in the development of more aggressive CRCs. In addition, multiplex analysis of TMAs together with immunohistochemistry (IHC) supplemented by hierarchical clustering are a useful, promising and very powerful tool for the identification of tumour subgroups with diagnostic and prognostic signatures.

Keywords: Cytokeratins, colorectal cancer (CRC), tissue microarray (TMA), prognosis, hierarchical clustering, epithelial–mesenchymal-transition (EMT)

1. Introduction

The cytoskeleton of epithelia is predominantly formed by cytokeratins (CK), which are grouped into a type I (acidic, *CK9* through *CK20*) and a type II (neutral-basic, *CK1–CK8*) gene family [10]. CKs provide mechanical stability to tissues, as implicated by a range of pathological phenotypes seen in patients bearing mutations in epidermal keratins [6]. All of the CKs share the same domain structure and form obligate heteropolymers from any combination of type I and II molecules to built intermediate filaments [8]. In various epithelia, they form specific expression pairs of at least one protein member of each type. In normal epithelium, luminal cells usually express *CK8*, *18*, and *19*, which is typical for simple epithelia [4,18]. Most malignant tumours are adenocarcinomas derived from simple epithelium, and monoclonal antibodies directed against *CK18* or in colorectal carcinoma against *CK20* have therefore been used to identify primary and metastatic cancer cells in numerous investigations [18].

Cancer of the colon and rectum is the second most prevalent cause of cancer deaths in men and the third most common in women [11]. Postoperative adjuvant chemotherapy improves the outcome in stage III (Dukes' stage C) colon cancer and is now widely accepted as standard therapy [9,17]. Many patients with stage II (Dukes' stage B) disease are considered to be at high risk for recurrence and receive adjuvant therapy, although its benefit in such cases is uncertain.

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Markers that reliably predict survival are needed [5, 19,12–14]. These biomarkers should support the clinical treatment of neoplastic processes, e.g. by selecting specific drug regimens.

Genome-wide expression profiling has identified a number of genes expressed at higher levels in colorectal carcinoma than in normal tissue, representing excellent candidates for diagnostic IHC. TMAs can be used to test the prognostic significance of antibodies against proteins encoded by differentially expressed genes using large numbers of archival patient specimens. We could recently identify CRC subgroups by IHC analysis of so far unknown biomarkers [12]. Our objectives in this study were (1) to test whether cytokeratins are differentially expressed in colorectal carcinomas on the protein level, (2) to evaluate these potential immunohistochemical markers in a series of well-characterised colorectal carcinomas including primary and metastatic tumours, and (3) by applying hierarchical cluster analysis to the semiquantitatively scored data to determine whether a panel of cytokeratin markers allows a meaningful grouping of the colorectal carcinomas.

2. Materials and methods

2.1. Tissue array construction

Two TMAs containing 468 samples from 203 patients were constructed. Tissue samples originated from surgical resections at the Departments of Surgery of the Charité. Ethical approval for the use of human tissue samples was obtained from the ethics Committee of the Charité University Hospital.

The tumour collective and its clinicopathological data are summarised in Table 1. One 0.6 mm core was taken from a representative area of the tumour and inserted into a recipient paraffin block to create the TMA [15]. We investigated serial slides cut consecutively and examined the same tumour region in multiplex immunohistochemical analysis of TMAs as previously described [12]. In total, 2919 specimens of colorectal tissue were evaluated.

Table 1
Cases used for Tissue Microarray (TMA)

Number of specimens	468
Number of patients	203
Primary tumours	154
Metastases	303
liver	74
lymph nodes	150
abdominal wall	60
lung	18
bone	1
Local recurrences	11

2.2. Immunohistochemistry

Commercial available antibodies against *CK5*, *7*, *8*, *13*, *14* and *CK16-20* were used. Antibody sources and staining conditions, including antigen retrieval methods, are summarised in Table 2. Antigen retrieval was performed in a pressure cooker by boiling for 5 minutes then incubating 25 minutes in citrate buffer. Slides were stained manually using the DAKO ChemMate TM Detection Kit Alkaline Phosphatase/Red Code No. K 5005 (Dako Corporation) following manufacturer's instructions. DAKO TBS (Tris-buffered saline) was used as washing buffer. For all antibodies the immunostaining of the cells was evaluated and scored semiquantitatively: 9, uninterpretable (missing spot, no tumour cells or uninterpretable staining); 0, negative; 1+, weak; 2+, moderate and 3+, strongly positive.

2.3. Hierarchical cluster analysis

Hierarchical cluster analysis of our TMA data was performed using the Cluster and TreeView software tools programs that were originally developed for analyzing cDNA microarray data (Gene Cluster 3.0 by Michel de Hoon, http://sourceforge.net/projects/jtreeview and http://rana.lbl.gov/EisenSoftware.htm). An Excel macro was designed for converting raw TMA staining data from a workbook with multiple worksheets in Excel, into a tabular format compatible for use with Gene Cluster. Average-linkage hierarchical clustering [7] was then performed on the reformatted data using the Cluster software, with filters set to require at least 80% interpretable immunostaining data for each specimen ($n = 278$) of 10 immunohistochemical evaluation methods (2780 datasets). Hierarchical clustering was carried out in two dimensions: tumours were grouped together based on the relatedness of their immunostaining profile, and antibodies were grouped based on which tumours they stain. The output was visualised using TreeView, which graphically displays the results of the cluster analysis as dendrograms and arrays, wherein the rows and columns correspond to the raw staining data, presented in the order determined by hierarchical clustering.

Table 2
Antibodies for immunohistochemistry

Antigen	Product no.	Supplier	Dilution	Pretreatment
Ck20	M 7019	DAKO	1:200	Microwave
Ck19	MU 246 UC	BioGenex	1:100	Microwave
Ck18	MU 143 UC	BioGenex	1:1000	Microwave
Ck17	M 7046	DAKO	1:20	Microwave
Ck16	NCL-CK16	Loxo/Novocastra	1:20	Microwave
Ck14	MU 146 UC	BioGenex	1:50	Microwave
Ck13	M 7003	DAKO	1:50	Microwave
Ck8	MU 142 UC	BioGenex	1:500	Microwave
Ck7	M 7018	DAKO	1:500	Microwave
Ck5	NCL-CK5	Loxo/Novocastra	1:50	Microwave

2.4. Statistical analysis

Fisher's exact test was used to determine the strength of association between all investigated clinicopathological parameters. P values $\leqslant 0.05$ were considered significant. All calculations were performed on a PC using the statistical software package SPSS (version 13, Munich, Germany). Clinicopathological parameters including follow-up were available for all specimens with a mean follow-up period of 108 weeks. The differences of the Kaplan–Meier survival curves were tested for statistical significance with the log rank test and the 95% confidence intervals were calculated. For each tumour specimen, the date of operation, date of last follow up, and vital status at last follow up (i.e., living or deceased) were recorded. Disease-specific survival was calculated.

Multivariate analyses were performed with a proportional hazard model (i.e. Cox regression) and stepwise backward/forward procedures provided by SPSS software were used to reduce the number of variables in the Cox models. For assessing and comparing the Cox models, a Wald test with significance level of 0.05 was used for both inclusion and exclusion of variables.

3. Results

3.1. Immunohistochemistry

In total, immunohistochemical data from 2919 colorectal tissue spots of colorectal cancer and normal colon mucosa was acquired using 10 different antibodies. The results of the entire tumour collective and all antibodies are summarised in Table 3. The expression was scored semiquantitatively by a 4-tier scale (0 – negative, 1 – weak, 2 – moderate, 3 – strongly positive, Fig. 1A) for the clustering analysis. This was reduced to a 2-tier system (0/1 – negative, 2/3 – positive) for the independently performed statistical analysis of single genes and their correlation with clinicopathological parameters including survival (Fig. 1B–E).

Table 3
Investigated specimens with immunohistochemical results

Evaluation method	All evaluated specimens ($n = 2919$)			
	Low ($n = 1681$)		High ($n = 1238$)	
	n	(%)	n	(%)
Ck20	63	(21)	237	(79)
Ck19	51	(16.5)	258	(83.5)
Ck18	12	(4.3)	269	(95.7)
Ck17	272	(98.6)	4	(1.4)
Ck16	278	(96.5)	10	(3.5)
Ck14	110	(40.3)	163	(59.7)
Ck13	273	(94.5)	16	(5.5)
Ck8	44	(14.8)	253	(85.2)
Ck7	282	(93.4)	20	(6.6)
Ck5	296	(97.4)	8	(2.6)

3.2. Hierarchical cluster analysis of tissue microarray immunostains

An unsupervised, hierarchical clustering algorithm allowed us to cluster the specimens on the basis of their similarities measured over the 10 immunohistochemical markers. Requiring 80% interpretable immunostaining results for each specimen, in total 2780 data points were included in the analysis. For each of the antibodies indicated at the top of the figure, strong positive staining is indicated by a red square, moderate positivity in dark brown, weak by light brown, absence of staining as black and no available data as grey. The expression of the antibodies was clustered

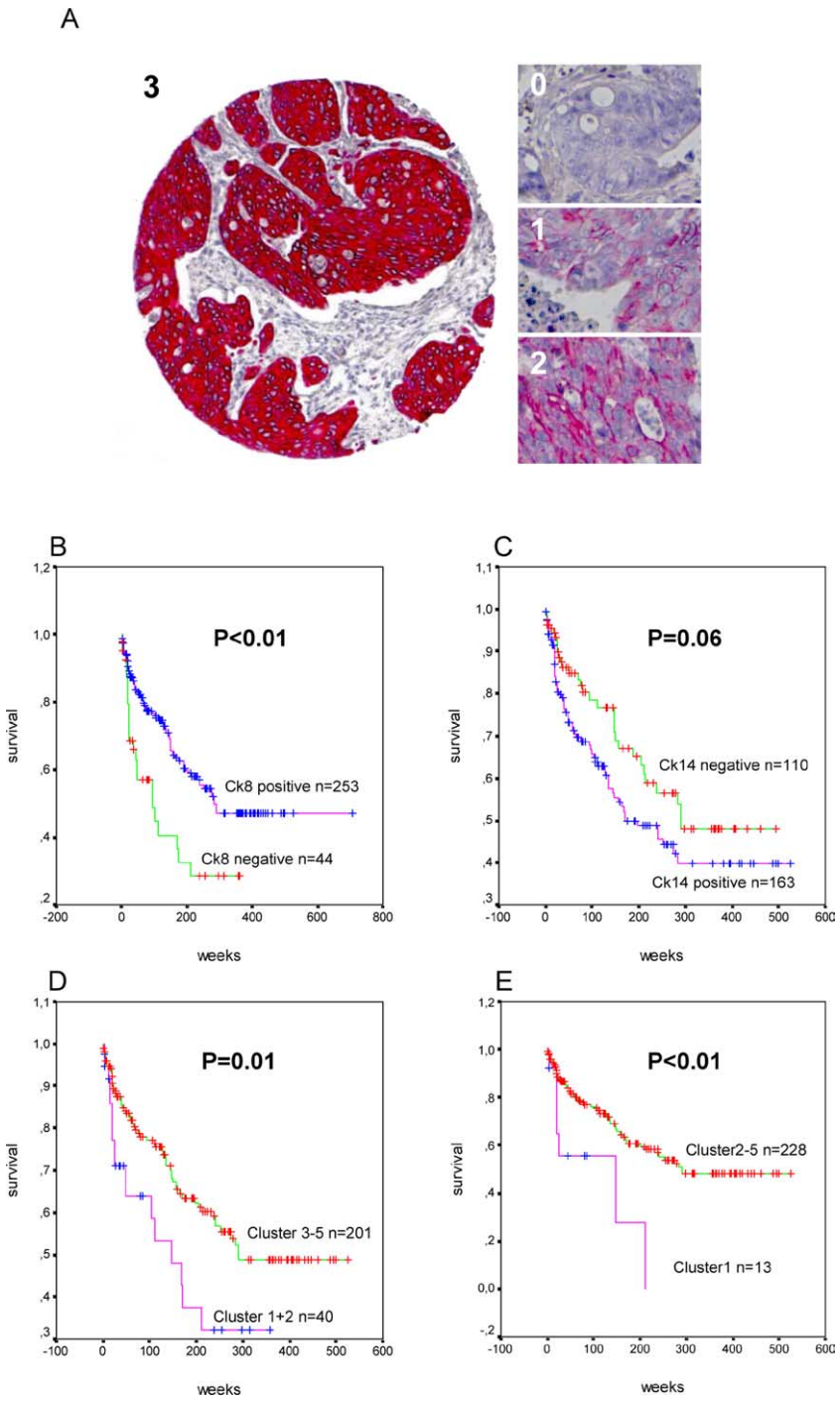

Fig. 1. (A) Examples of the immunohistochemical assessment of Cytokeratin 8 (CK8) staining in colorectal carcinomas using TMAs. Negative staining of tumour cells (0), weakly positive (1), moderately positive (2), strongly positive (3) (50× magnification). (B) Kaplan–Meier plot comparing disease-specific survival in patients with CK8-positive colorectal tumours ($n = 253$) and patients with CK8-negative tumours ($n = 44$), $p < 0.01$. (C) Kaplan–Meier plot comparing disease-specific survival in patients with CK14-positive colorectal tumours ($n = 163$) and patients with CK14-negative tumours ($n = 110$), $p = 0.06$. (D) Kaplan–Meier plot comparing disease-specific survival in Cluster 1 and 2 "shorter survival clusters" with Clusters 3–5, $p = 0.01$. (E) Kaplan–Meier plot comparing disease-specific survival in Cluster 1 "extreme shorter survival cluster" with 2–5, $p < 0.01$.

Table 4
Immunohistochemical results with cytokeratin 8, 14 and 20 in colorectal carcinoma

	CK8 $N = 297$				CK14 $N = 275$				CK20 $N = 300$			
	CK8 low		CK8 high		CK14 low		CK14 high		CK20 low		CK20 high	
	N (%)		N (%)		N (%)		N (%)		N (%)		N (%)	
	44	(15)	253	(85)	110	(40)	163	(60)	62	(21)	237	(79)
Primary tumour	14	(12)	99	(88)	30	(30)	71	(70)	20	(18)	89	(82)
Metastasis	30	(16)	154	(84)	80	(47)	92	(53)	42	(22)	148	(78)
liver	8		43		24		24		13		35	
lymph nodes	13		68		31		47		21		66	
abdominal wall	7		31		22		11		6		36	
lung	1		12		3		9		2		10	
bone	1		0		0		1		0		1	
Tumour infiltration												
pT1/2	3	(1)	44	(15)	5	(6)	29	(12)	11	(4)	37	(12)
pT3/4	41	(14)	207	(70)	5	(38)	131	(52)	52	(18)	195	(66)
Tumour differentiation												
G1/G2	28	(10)	183	(65)	73	(28)	115	(44)	44	(16)	164	(58)
G3	14	(5)	57	(20)	33	(13)	38	(15)	11	(4)	63	(22)
Nodal status												
pN0	8	(3)	46	(16)	17	(6)	31	(12)	11	(4)	40	(14)
pN1/N2	34	(11)	204	(70)	90	(34)	130	(48)	52	(17)	190	(65)
Metastasis												
M0	10	(3)	94	(32)	36	(13)	59	(22)	22	(7)	88	(30)
M1	34	(12)	159	(53)	74	(27)	103	(38)	41	(14)	146	(49)

on the basis of their similarities measured over the group of 278 tumours (Fig. 2A). In the dendrograms shown in Fig. 2, the length and the subdivision of the branches displays the relatedness of the colorectal tumours (left) and the gene expressions (top). In the first step, unsupervised hierarchical clustering was applied to the dataset of 10 markers. As expected, different cytokeratins clustered on the right side (top), however, the tumour groups were not very well discriminated (left, Fig. 2A). Therefore, six markers, i.e. *CK5*, *CK7*, *CK13*, *CK16*, *CK17*, and *CK18*, discriminating less than five percent of the tumours with low or high expression were excluded. With the remaining four biomarkers *CK20*, *CK19*, *CK8*, and *CK14* a clear separation of the specimens into five distinct groups with large linkage distances became apparent (Fig. 2B). Cluster group 1 (yellow) was dominated by negative expression of *CK20*, *CK19*, and *CK8*, whereas cluster group 2 (orange) was separated mainly by negative *CK8* staining alone (Fig. 2B,C). The other subgroups where distinguished by the high expression of *CK8* (Clusters 3–5), additionally group 4 (blue) by low expression of *CK20* and group 5 (black) by low expression of *CK19*.

3.3. Immunohistochemistry and clinicopathological parameters

Survival analysis Exploratory analysis was conducted to correlate the outcome of patients monitored during the 706-week period with the immunohistochemistry results. The analysis was restricted to disease specific survival and was performed on all specimens. Overexpression (score 2+ and 3+) of *CK8* was found in 253 (85.2%) specimens. 44 (14.8%) exhibited no relevant *CK8* staining (score 0 and 1+). The corresponding survival curves according to *CK8* expression are shown in Fig. 1B. The median survival time of the patients was 189 weeks. Statistical analysis showed that patients with high *CK8* expression tumours had significantly longer survival rates than patients with low *CK8* expression ($p = 0.001$). The high *CK8* expression was also correlated with a longer patients survival including only the primary tumours ($p = 0.02$). The fact that this analysis carried a lower value of significance than the analysis including the metastases is probably related to the fact that the metastases represent the more aggressive tumour cell clones. Overexpression of *CK14* in 163 specimens (59.3%) showed a trend to a shorter patients survival ($p = 0.06$). All other

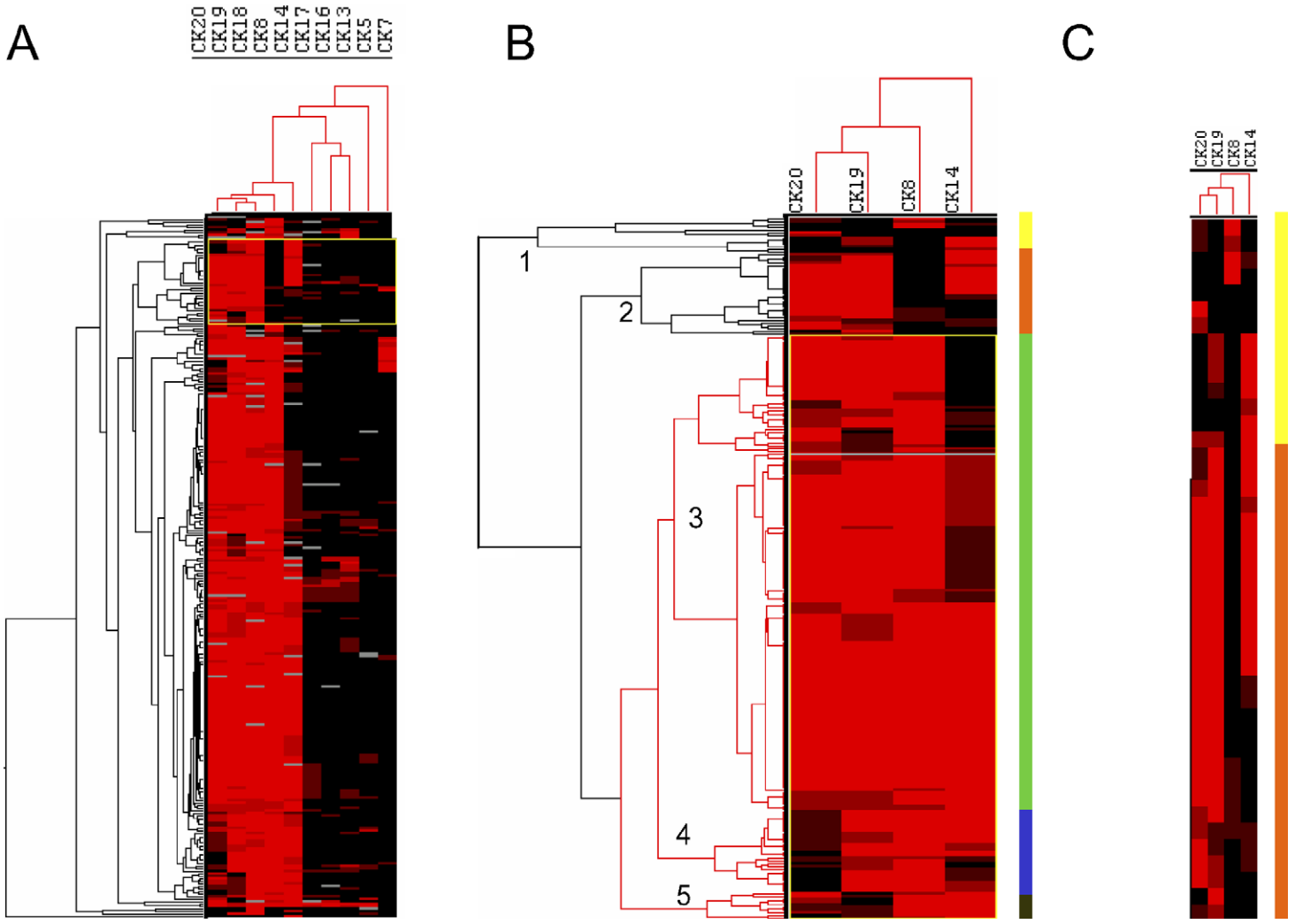

Fig. 2. (A) Hierarchical cluster analysis of colorectal carcinoma tissue microarray immunostaining results. For each of the antibodies indicated at the top of the figure, strong positive staining is indicated by a red square, moderate staining by a light brown, weak positive by dark brown, absence of staining as black, and no available data as grey. The dendogram at the top shows the clustering of antibodies based on the relatedness of tumours stained by each antibody. The dendogram on the left side shows the clustering of the tumours based on the degree of similarity of their immunohistochemical staining results. (B) Cluster analysis with cytokeratin 20, 19, 8 and 14. (C) Enlarged portion from Cluster 1 (yellow), the so called "extreme shorter survival cluster" with reduced CK20 and CK8 staining (see vertical line) and cluster 2 (orange), together the "shorter survival cluster" with prominently reduced CK8 staining (see vertical line). Number and different colors: cluster group 1 (yellow), 2 (orange), 3 (green), 4 (blue), 5 (black).

investigated antigens showed no prognostic relevance ($p > 0.05$).

Comparing cluster group 1 and cluster group 2 with the other cluster groups, we could demonstrate a significant difference ($p = 0.01$) with shorter survival in cluster 1 and 2 (see Fig. 1D). Comparing cluster group 1 with the others the p-value was even more significant ($p < 0.01$) (Fig. 1E).

TNM parameters Reduced *CK5* expression (score 0 and 1+) showed a trend to a higher tumour stage (pT1/2 versus pT3/4, $p = 0.05$), but could not reach significance. No other significant correlation with antibody expression could be demonstrated. The comparison between the primary tumours and the metastases did not show significant differences. When performing multivariate analysis, comparing *CK8* with the parameters' tumour stages (pT), grading (G), and nodal stage (pN), *CK8* was not an independent parameter, nethertheless showing higher significance than the nodal status. Typical expression patterns of individual genes are available as supplementary data on our Berlin-TMA-web-portal http://pathoweb.charite.de/tmaportal.

4. Discussion

This study is the first comprehensive and largest analysis of different cytokeratins associated with clinicopathological parameters in colorectal carcinomas (CRCs) using the synergy of tissue microarray (TMA) and hierarchical clustering. We were able to investigate 2919 specimens. As a result, new biomarkers and signatures in the progression of colorectal cancer (CRC) were detected.

It is proposed that epithelial cell subpopulations actively downregulate cytoskeleton proteins, e.g. cytokeratins and cell–cell adhesion molecules, during embryogenesis and leave their "local neighborhood" to move into new microenvironments where they differentiate into distinct cell types [20]. This regulated phenotypic modulation is called epithelial–mesenchymal transition (EMT) and occurs, for example, during gastrulation and neural crest cell migration. In cancer, it is also assumed that dedifferentiation of tumour cells in a mesenchymal phenotype occurs in malignant progression and initiate metastasis [1,16]. In this study we investigated for the first time different cytokeratins in a large amount of colorectal cancer specimens of a well characterised tumour collectice with multiplex analysis of TMAs which is in our opinion a very good approach to analyze tumour cells from the same clone [12].

Unsupervised, hierarchical clustering allowed us to subgroup 287 colorectal cancer specimens on the basis of their similarities in gene expression (Fig. 2). We were able to distinguish colorectal cancer specimens in five groups with the help of hierarchical clustering. Clear separation of the specimens with large linkage distances was detected when clustering with four biomarkers, *CK20*, *CK19*, *CK14* and *CK8* (Fig. 2B). Notably, in cluster 1 and 2, the tumours a significantly linked to a shorter survival ($p = 0.01$) and 90 percent of the tumours were CK8 negative, thus representing "shorter survival cluster". Cluster 1 itself, the so called "extreme shorter survival cluster", showed even a higher significant value ($p < 0.01$) with shorter patients survival, which is represented by reduced *CK20* (85 percent) and *CK8* (71 percent) staining. This may indicate that the loss of *CK8* and *CK20* is an important event in EMT. It is important to note that for diagnostic procedures *CK20* is used as a colon carcinoma marker protein in the detection of metastasis of unknown primary tumours. However, although *CK20* still detects 90 percent of colorectal carcinoma cells in our study, a specific subgroup of *CK20* negative tumours was located in the extreme short survival cluster and represents a subtype of a specific cell clone with a very aggressive potential. Using *CK20* as a colon carcinoma marker in the detection of metastasis of unknown primary tumours, it is important to know, that very aggressive tumour cell clones may loose *CK20* expression and are not detectable with this cytokeratin. *CK20* expression might be variable within the tumour, but it is meanwhile accepted that the analysis of a high number of overall samples by TMA compensate for this potential bias of intratumour heterogeneity [2].

To the best of our knowledge, this is the first study showing that reduced *CK8* expression is significantly associated with a shorter patients' survival in CRC ($p < 0.01$). Interestingly, our results are in concordance with observations in breast cancer [22], also showing that relapse-free survival for patients with *CK8* positive tumours was significantly better than that for patients with *CK8* negative tumours. Additionally, Schaller showed that elevated *CK18* protein expression, which is also a luminal epithelial cytokeratin usually coexpressed with *CK8*, indicating a favorable prognosis in patients with breast cancer [21]. Recently it could be shown that transfection of the *CK18* gene in human breast cancer cells causes induction of adhesion proteins and a dramatic regression of malignancy

in vitro and *in vivo* [3]. We speculate that the presence of specific cytokeratins, in our study *CK8* in CRC, might stabilise malignant cells in its cytoskeleton antagonizing tumour progression or metastasis.

On the other side, cytokeratin 14 is regarded as a basal cell marker of squamous and glandular epithelia and is often coexpressed with *CK5* which is regarded as a stem cell or progenitor cell marker. Interestingly, in our study overexpression of *CK14* showed a trend to a shorter survival ($p = 0.06$) and *CK5* a trend to a higher tumour infiltration ($p = 0.05$), showing that different cytokeratins are associated with different functions in carcinogenesis of CRC, e.g. involved in a more aggressive phenotype. The progenitor or stem cell markers including *CK5/14* need to be evaluated in further molecular studies and are the topic in many research groups at this time.

In conclusion, in this study we show that reduced *CK8* expression in CRC is significantly associated with shorter patients' survival and that a specific subgroup of *CK8* negative CRCs which are also *CK20* negative have an even more significant correlation with shorter patients' survival. These results show that *CK8* and *CK20* are very important cytokeratins in CRC with the highest diagnostic and prognostic relevance. Furthermore, we were able to show that the synergy of hierarchical clustering and TMA immunohistochemistry, i.e. the combination of a high throughput technology with an elegant statistical method, is a useful, promising and very powerful tool for further investigations being able to decipher diagnostic and prognostic signatures of cancer subtypes.

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