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.
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.

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.

| 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  |
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 \( \leq 0.05 \) 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).

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, $p < 0.01$. 

T. Knösel et al. / Cytokeratin profiles in multiplex analysis of TMAs in CRC
Table 4

| Gene  | N (%)  | N (%)  | N (%)  | N (%)  | N (%)  | N (%)  |
|-------|--------|--------|--------|--------|--------|--------|
| CK8 low | 44 (15) | 253 (85) | 30 (30) | 71 (70) | 20 (18) | 89 (82) |
| CK8 high | 253 (85) | 110 (40) | 163 (60) | 62 (21) | 237 (79) |
| CK14 low | 30 (30) | 71 (70) | 42 (22) | 148 (78) |
| CK14 high | 71 (70) | 62 (21) | 237 (79) |  |
| CK20 low | 20 (18) | 89 (82) | 148 (78) |  |
| CK20 high | 89 (82) | 237 (79) |  |

Primary tumour
- CK8 low: 44 (15)
- CK8 high: 253 (85)
- CK14 low: 30 (30)
- CK14 high: 71 (70)
- CK20 low: 20 (18)
- CK20 high: 89 (82)

Metastasis
- Liver: 8 (43)
- Lymph nodes: 13 (68)
- Abdominal wall: 7 (31)
- Lung: 1 (12)
- Bone: 1 (0)

Tumour infiltration
- pT1/2: 3 (14)
- pT3/4: 41 (14)

Tumour differentiation
- G1/G2: 28 (10)
- G3: 14 (5)

Nodal status
- pN0: 8 (4)
- pN1/N2: 34 (14)

Metastasis
- M0: 10 (3)
- M1: 34 (12)

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 below represents the clustering of 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 signif-
ificant 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 an-
tibody expression could be demonstrated. The com-
parison between the primary tumours and the metas-
tases did not show significant differences. When per-
forming multivariate analysis, comparing CK8 with
the parameters’ tumour stages (pT), grading (G), and
nodal stage (pN), CK8 was not an independent para-
meter, nethertheless showing higher significance than
the nodal status. Typical expression patterns of individ-
ual 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 clin-
icopathological 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 sig-
natures in the progression of colorectal cancer (CRC)
were detected.

It is proposed that epithelial cell subpopulations ac-
tively downregulate cytoskeleton proteins, e.g. cyto-
keratins and cell–cell adhesion molecules, during em-
byogenesis and leave their “local neighborhood” to
move into new microenvironments where they differ-
entiate into distinct cell types [20]. This regulated pheno-
typic modulation is called epithelial–mesenchymal
transition (EMT) and occurs, for example, during gas-
trulation and neural crest cell migration. In cancer, it
is also assumed that dedifferentiation of tumour cells
in a mesenchymal phenotype occurs in malignant pro-
gression 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 ba-
sis 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 bio-
markers, CK20, CK19, CK14 and CK8 (Fig. 2B). Not-
bly, in cluster 1 and 2, the tumours a significantly
linked to a shorter survival \((p = 0.01)\) and 90 per-
cent of the tumours were CK8 negative, thus represent-
ing “shorter survival cluster”. Cluster 1 itself, the so
called “extreme shorter survival cluster”, showed even
a higher significant value \((p < 0.01)\) with shorter pa-

tients survival, which is represented by reduced CK20
\((85\%\) and CK8 \((71\%\) staining. This may indi-
cate that the loss of CK8 and CK20 is an important

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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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References

[1] T. Brabletz, F. Hlubek, S. Spaderna, O. Schmalhofer, E. Hiendlmeyer, A. Jung and T. Kirchner, Invasion and metastasis in colorectal cancer: epithelial–mesenchymal transition, mesenchymal–epithelial transition, stem cells and beta-catenin, Cells Tissues Organs 179 (2005), 56–65.

[2] L. Babendorf, A. Nocito, H. Moch and G. Sauter, Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies, J. Pathol. 195 (1) (2001), 72–79.

[3] H. Buhler and G. Schaller, Transfection of keratin 18 gene in human breast cancer cells causes induction of adhesion proteins and dramatic regression of malignancy in vitro and in vivo, Mol. Cancer Res. 3 (2005), 365–371.

[4] P.G. Chu and L.M. Weiss, Keratin expression in human tissues and neoplasms, Histopathology 40 (2002), 403–439.

[5] D.C. Chung, Molecular prognostic markers and colorectal cancer: The search goes on, Gastroenterology 114 (1998), 1330–1332.

[6] P.A. Coulombe, M.E. Hutton, A. Letai, A. Hebert, A.S. Paller and E. Fuchs, Point mutations in human keratin 14 genes of epidermolysis bullosa simplex patients: genetic and functional analyses, Cell 66 (1991), 1301–1311.

[7] M.B. Eisen, P.T. Spellman, P.O. Brown and D. Botstein, Cluster analysis and display of genome-wide expression patterns, Proc. Natl. Acad. Sci. USA 95 (1998), 14863–14868.

[8] E. Fuchs and D.W. Cleveland, A structural scaffolding of intermediate filaments in health and disease, Science 279 (1998), 514–519.

[9] E. Galanis, S.R. Alberts and M.J. O’Connel, New adjuvant therapy for colon cancer: justified hope or commercial hype, Surg. Oncol. Clin. North Am. 9 (2000), 813–823.

[10] M. Hesse, T.M. Magin and K. Weber, Genes for intermediate filament proteins and the draft sequence of the human genome: novel keratin genes and a surprisingly high number of pseudo-genes related to keratin genes 8 and 18, J. Cell Sci. 114 (2001), 2569–2565.

[11] A. Jemal, A. Thomas, T. Murray and M. Thun, Cancer statistics 2002, CA Cancer J. Clin. 52 (2002), 23–47.

[12] T. Knösel, A. Emde, K. Schlüns, Y. Chen, K. Jürchott, M. Krause, M. Dietel and I. Petersen, Immunoprofiles of 11 biomarkers using tissue microarrays identify prognostic subgroups in colorectal cancer, Neoplasia 7 (2005), 741–747.

[13] T. Knösel, K. Schlüns, U. Stein, H. Schwabe, P.M. Schlag, M. Dietel and I. Petersen, Genetic imbalances with impact on survival in colorectal cancer patients, Histopathology 43 (2003) 323–331.

[14] T. Knösel, Y. Yu, U. Stein, H. Schwabe, K. Schlüns, P.M. Schlag, M. Dietel and I. Petersen, Overexpression of cyclooxygenase-2 correlates with chromosomal gain at the cyclooxygenase-2 locus and decreased patient survival in advanced colorectal carcinomas, Dis. Colon Rectum. 47 (2004), 70–77.

[15] J. Kononen, L. Babendorf, A. Kallioniemi, M. Barlund, P. Schraml, S. Leighton, J. Torhorst, M.J. Mihatsch, G. Sauter and O.P. Kallioniemi, Tissue microarrays for high-throughput molecular profiling of tumour specimens, Nat. Med. 4 (1998), 844–847.

[16] E. Korschig, J. Puckeisen, C. Liesdte, D. Hungermann, P. Wütling, P.J. van Diest, B. Brandt, W. Bockeller and H. Buerger, The origin of vimentin expression in invasive breast cancer: epithelial–mesenchymal transition, myoepithelial histogenesis or histogenesis from progenitor cells with bilinear differentiation potential?, J. Pathol. 206 (2005), 451–457.
[17] J.S. Macdonald, Adjuvant therapy of colon cancer, CA Cancer J. Clin. 49 (1999), 202–219.

[18] R. Moll, W.W. Franke, D.L. Schiller, B. Geiger and R. Krepler, The catalog of human cytokeratins: patterns of expression in normal epithelia, tumours, and cultured cells, Cell 31 (1982), 11–24.

[19] M.J. O’Connel, D.J. Schaid, V. Ganju, J. Cunningham, J.S. Kovach and S.N. Thibodeau, Current status of adjuvant chemotherapy for colorectal cancer. Can molecular markers play a role in predicting prognosis?, Cancer 70 Suppl. (1992), 1732–1739.

[20] P. Savagner, Leaving the neighborhood: molecular mechanisms involved during epithelial–mesenchymal transition, Bioessays 23 (2001), 912–923.

[21] G. Schaller, I. Fuchs, W. Pritze, A. Ebert, H. Herbst, K. Pantel, H. Weitzel and E. Lengyel, Elevated keratin 18 protein expression indicates a favorable prognosis in patients with breast cancer, Clin. Cancer Res. 2 (1996), 1879–1885.

[22] H. Takei, Y. Iino, J. Horiguchi, T. Kanoh, Y. Takao, T. Oyama and Y. Morishita, Immunohistochemical analysis of cytokeratin #8 as a prognostic factor in invasive breast carcinoma, Anticancer Res. 15 (1995), 1101–1105.