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ORIGINAL ARTICLE

Molecular profiles and clinical outcome of stage UICC II colon cancer patients

Jörn Gröne · Dido Lenze · Vindi Jurinovic · Manuela Hummel · Henrik Seidel · Gabriele Leder · Georg Beckmann · Anette Sommer · Robert Grützmann · Christian Pilarsky · Ulrich Mansmann · Heinz-Johannes Buhr · Harald Stein · Michael Hummel

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Abstract
Purpose Published multigene classifiers suggesting outcome prediction for patients with stage UICC II colon cancer have not been translated into a clinical application so far. Therefore, we aimed at validating own and published gene expression signatures employing methods which enable their reconstruction in routine diagnostic specimens.
Methods Immunohistochemistry was applied to 68 stage UICC II colon cancers to determine the protein expression of previously published prognostic classifier genes (CDH17, LAT, CA2, EMR3, and TNFRSF11A). RNA from macrodissected tumor samples from 53 of these 68 patients was profiled on Affymetrix GeneChips (HG-U133 Plus 2.0). Prognostic signatures were generated by "nearest shrunken centroids" with cross-validation. Previously published gene signatures were applied to our data set using "global tests" and leave-one-out cross-validation
Results Correlation of protein expression with clinical outcome failed to separate patients with disease-free follow-up (group DF) and relapse (group R). Although gene expression profiling allowed the identification of differentially expressed genes ("DF" vs. "R"), a stable classification/prognosis signature was not discernable. Furthermore, the application of previously published gene signatures to our data was unable to predict clinical outcome (prediction rate 75.5% and 64.2%; n.s.). T-stage was the only independent prognostic factor for relapse with established clinical and pathological parameters including microsatellite status (multivariate analysis).
Conclusions Our protein and gene expression analyses do not support application of molecular classifiers for prediction of clinical outcome in current routine diagnostic as a basis for patient-orientated therapy in stage UICC II colon cancer. Further studies are needed to develop prognosis signatures applicable in patient care.

Keywords Colon cancer · Immunohistochemistry · Gene expression signature · Prognosis

J. Gröne (✉) · H.-J. Buhr
Department of General, Vascular and Thoracic Surgery, Charité University Medicine Berlin,
Campus Benjamin Franklin, Hindenburgdamm 30,
12200 Berlin, Germany
e-mail: joern.groene@charite.de

D. Lenze · H. Stein · M. Hummel
Institute of Pathology, Charité University Medicine Berlin,
Campus Benjamin Franklin, Hindenburgdamm 30,
12200 Berlin, Germany

V. Jurinovic · U. Mansmann
Institut für Medizinische Informatik Biometrie Epidemiologie (IBE),
Munich, Germany

M. Hummel
Core Facilities-Microarray Unit, Centre for Genomic Regulation,
C/Dr. Aiguader 88,
08003 Barcelona, Spain

H. Seidel · G. Leder · G. Beckmann · A. Sommer
Target Discovery, Bayer Schering Pharma AG,
Müllerstr. 178,
13353 Berlin, Germany

R. Grützmann · C. Pilarsky
Department of Visceral, Thoracic and Vascular Surgery,
University Hospital Dresden,
Fetscherstr. 74,
01307 Dresden, Germany

Introduction

Therapy concepts of solid cancers changed from monotherapy to multimodal treatment regimes within the last decades due to poor prognosis. Tailored concepts comprising radiation and chemotherapy for distinct patient groups have shown to improve prognosis of the respective patients. Despite these documented advantages, a proportion of patients does not benefit and, moreover, suffers from side effects of multimodal treatment modalities. On the ambitious way to individualized therapy of cancer patients, a multitude of efforts has been undertaken to identify subgroups with a high chance of response or high susceptibility for relapse [1–4].

Colorectal cancer, the second most common cancer type in industrialized nations, is a good example for multimodal and sophisticated curative treatment regimes. Both colon and rectal cancers are primarily treated by surgery, which is supplemented with neoadjuvant and adjuvant (post-surgery) therapy depending on stage and localization. In locally advanced colon cancer (nodal positive; UICC III), adjuvant chemotherapy is recommended to all stage III patients, increasing the 5-year disease-free survival from 48% to 65% [5]. In stage UICC II disease, up to 25% of all resected patients develop relapse and die from the disease within 5 years. Unfortunately, identification of these UICC II patients is—based on currently available methods—not possible, and application of adjuvant chemotherapy to all UICC II patients has no significant effect on disease-free survival (i.e., from 72% to 76%) and overall survival (i.e., from 80% to 81%) [5]. In addition, administration of adjuvant chemotherapy to all stage II patients is unnecessary and harmful for three fourths of patients which are cured by surgery alone. Therefore, the identification of UICC II patients with a high risk of tumor relapse that might benefit from adjuvant treatment is highly desirable.

Gene expression signatures have been suggested as promising tools to identify patients who suffer from disease with the same pathological stages and comparable clinical features but with different clinical outcome in solid and hematological cancers [6–17]. However, the overlap between gene signatures, even when established from the same disease and pathological stage, has been poor, and identification of robust multigene signature is still aggravated by small sample size, probe differences, and different platforms [18–20].

In stage UICC II colon cancer, four independent molecular signatures have been reported to predict the prognosis reaching 76.3% to 84% accuracy [21–23]. However, gene expression profiling by microarrays is technically elaborative, expensive, not widely available, and not applicable to routine diagnostic specimens. Therefore, a classifier based on methods suitable for routine diagnostic purposes is required. We aimed to establish a protein expression prognosticator that is applicable by means of immunohistochemistry using genes described to be members of published gene expression signatures. Since this approach was not satisfactory, we performed own gene expression profiling to validate previously published prognostic signatures in an independent cohort and to search for possible further prognostic signatures.

Methods

Patient characteristics and tumor samples

The study was approved by the local ethical committee. Tumor samples of 68 patients with sporadic stage UICC II colon cancer treated by elective standard oncological resection at the Department of General, Vascular and Thoracic Surgery, Campus Benjamin Franklin, Charité-Universitätsmedizin Berlin were collected after informed consent. A complete 5-year follow-up was available for all patients. None of the patients received adjuvant chemotherapy. Patients with proven hereditary colorectal cancer or inflammatory bowel disease were excluded from this study. Routine histopathologic staging of resected specimen was performed by experienced pathologists (hematoxylin and eosin staining (H&E)) comprising local tumor invasion (T), involvement of lymph nodes (N), and tumor differentiation (G1–3).

For analysis of microsatellite instability, genomic DNA from tumor and normal colon mucosa of each patient was isolated from frozen or formalin-fixed and paraffin-embedded (FFPE) tissue sections using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocols. Quantity and purity of the extracted DNA were assessed by photometric measurement (Nanodrop ND-1000, Nanodrop Technologies, USA). To evaluate the microsatellite status of the tumors in comparison to the matched normal tissue, the MSI Analysis System, Version 1.2 (Promega, USA) was used according to the manufacturer's instructions, except that POP-7 Polymer (Applied Biosystems, USA) was used for capillary electrophoresis on the Genetic Analyzer 3130 (Applied Biosystems, USA). A tumor sample was defined as microsatellite instable (MSI) when two or more of the analyzed markers were altered in comparison to the corresponding normal tissue.

Paraffin-embedded and formalin-fixed tumor tissue blocks from all 68 patients (the "protein collection") were available for immunohistochemistry. Corresponding transmural cancer specimens from 53/68 patients for RNA analysis (the "RNA collection") were collected and snap frozen at time of surgery and stored at −80°C. Patient and tumor characteristics are given in Table 1.

Table 1 Patient data

	Disease free (DF)		Relapse (R)		*p* value	
	IHC (n=54)	RNA (n=40)	IHC (n=14)	RNA (n=13)	IHC	RNA
Age at time of diagnosis (years)					0.939	0.569
Mean	67.13	66.15	65.57	63.23		
Sex					1	1
Female	27	20	7	7		
Male	27	20	7	6		
Localization of tumor					0.109	0.241
Coecum+colon ascendens	29	22	4	2		
Colon transversum+flexures	6	3	3	3		
Colon descendens+sigmoideum	19	15	7	8		
T-stage					0.015	0.001
T3	51	37	8	8		
T4	3	3	6	5		
Grading					0.246	0.164
Well/moderate (G1/G2)	36	26	12	11		
Low (G3)	18	14	2	2		
Microsatellite status[a]					0.334	0.317
Low	37	24	12	10		
High	17	16	2	3		

Clinical and histopathological characteristics of patients with sporadic stage UICC II colon cancer who underwent standard oncological resection (standard and extended right and left hemicolectomy with central ligature and lymphadenectomy). None of the patients received adjuvant chemotherapy

[a] According to Bethesda guidelines [40] (MSI-high: alteration of ≥2 of five markers, MSI low: alteration of ≤1 of five markers).

Immunohistochemistry

Protein selection The criteria used for selection of prognostic proteins were as follows: (a) the gene is part of the combined published prognostic signatures (n=77 genes), (b) the protein product of the respective gene is annotated, (c) known expression in colorectal tissue, (d) association with carcinogenesis, particularly in colorectal cancer, (e) availability of antibodies, and (f) positive correlation of RNA and protein expression.

Staining Sections from formalin-fixed and paraffin-embedded tissues were deparaffinized, and antigenicity was retrieved by cooking in citrate buffer (100 mM) for 5 min (TNFRSF11A antibody) or 2 min (all other antibodies). Stainings were performed on 68 cases employing mouse monoclonal antibodies against CDH17 (1:2,000; R&D Systems, USA), LAT (1:100; Dako, Denmark), CA2 (1:1,000; Santa Cruz), and TNFRSF11A (1:2,000; R&D Systems, USA), and rabbit polyclonal antibodies against EMR3 (1:1,000; Abcam, UK) and PBK (1:200; Abcam, UK). Binding was visualized directly with the APAAP Mouse REAL™ Detection System (Dako, Denmark) for antibodies against CDH17, LAT, and CA2. To detect binding of anti-EMR3 and anti-PBK antibodies, a mouse anti-rabbit bridging antibody (Dako, Denmark) was applied prior to the APAAP detection kit. Staining of TNFRSF11A became visible by application of the REAL™ Detection System, Alkaline Phosphatase/RED (Dako, Denmark).

Scores and analyses CDH17 and TNFRSF11A are expressed by the tumor cells and show a heterogeneous staining pattern in respect to the number of positive tumor cells as well as the intensity of the staining. We therefore applied the semiquantitative immunoreactivity score (IRS) as described by Noske et al. [24] in order to integrate both aspects. Briefly, the percentage of stained tumor cells (score 0–4) was multiplied with the staining intensity (score 0–3) to give the IRS score of each case (score 0–12). Cases with an IRS from 0–3 were combined to a CDH17- or TNFRSF11A-negative group, cases with an IRS from 4–7 were pooled as moderately CDH17- or TNFRSF11A-expressing samples, and cases with an IRS from 8–12 constitute the CDH17- or TNFRSF11A-positive group, respectively.

In contrast, LAT and EMR3 expressions were restricted to tumor-infiltrating immune cells, and the staining of these proteins was each scored by the number of positive cells (1= low number, 2= moderate number, 3=high number of cells) within the tumor cell area. CA2 staining was restricted to normal colon mucosa and therefore not scored. The staining for PBK was inconsistent despite the application of several commercially available antibodies. Therefore, the results of the PBK immunostainings, which were assessed as described for LAT and EMR3, were not considered.

Statistical analysis We used the Wilcoxon test to test for the difference in age between the two groups, and the χ^2 test for the parameter grading. The remaining parameters were tested with Fisher's exact test. For correlation of protein and RNA expression with clinical and pathological

parameters (age, gender, tumor localization, grading, T-stage, microsatellite status), available scores were then tested in multivariate Cox regression analysis. Correlation of expression of selected proteins (CDH17 and EMR3, one probe set each; TNFRSF11A and LAT, two probe sets each) and corresponding RNA expression data was demonstrated by scatter plots.

Microarray analyses

Tumor sample preparation and array hybridization For microarray analyses, snap frozen tissue specimens were cut into 7-μm-thick sections that were stained with H&E. Stained sections were reviewed by a pathologist to identify areas of vital tumor cells and to ensure a tumor content of 80–90%. Corresponding tumor areas were macrodissected by vertical 3-mm incision into the frozen tissue with a sterile blade. Incision was followed by a series of ten 20-μm frozen sections. Separated tumor areas were harvested by sterile micropipette tip and collected in buffer (RLT buffer, RNeasy Mini Kit; Qiagen, Hilden, Germany). Each series of ten sections was followed by a 7-μm H&E-stained section to control tissue composition. The number of tissue sections used to extract RNA was dependent on the expanse of the area of individual tumor tissue.

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and quantified using the Nanodrop ND-1000 UV–vis spectrophotometer (Nanodrop Technologies, USA). The quality of the RNA was controlled using the BioAnalyzer (Agilent Technologies, USA), and exclusively high quality RNA (RIN≥7.6) was used for further analysis. For Affymetrix GeneChip analysis, 3 μg total RNA of each sample was converted to biotin-labeled cRNA and hybridized on HG-U133 Plus 2.0 arrays (Affymetrix, USA), following the manufacturer's recommendations.

Microarray data analysis The quality of all microarrays was reviewed by inspection of scatter plots (MvA plots) [25]. Variation of non-biological origin between the arrays were reduced by normalization (variance stabilization) using the *vsn* package in R (language and environment for statistical computing and graphics). "vsn" is a robust method for normalization of large-scale gene expression data. When running experiments that involve multiple high-density oligonucleotide arrays, it is important to remove sources of variation between arrays of non-biological origin. Normalization is a process for reducing this variation that works also on values that are negative after background subtraction [10]. For construction of a classifier for relapse (yes/no), the method of "nearest shrunken centroids" was applied [26] based on all stage UICC II patients and on the subgroup of microsatellite stable (MSS) patients. To avoid overfitting, a repeated double cross-validation procedure was used [27]. The data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE18088 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hncvtygaygqmghg&acc=GSE18088).

Data of previously published prognostic gene expression signatures involving patients with stage UICC II colon cancer were analyzed by testing their power to separate between patients with relapse or disease-free patients in our data set using "global test." This test can determine whether the global expression pattern of a group of genes is significantly related to clinical variable [28] (Table 2). The two data sets of Lin et al. [22] were validated as published by the authors (New Zealand data: support vector machine; German data set: three nearest neighbor classificator, leave-one-out cross-validation, permutation approach).

Results

Our study comprised paraffin-embedded and formalin-fixed tissues from 68 patients all of which have been employed for immunohistochemistry (IHC) detection of protein expression ("protein collection"). In addition, frozen tissue specimens were available for 53 of these 68 patients (78%).

Table 2 Published signatures

Study	Dataset	Size of signature	Number of corresponding probe sets	*p* value
Wang et al. (2004) [23]		23	23 Affymetrix probe sets	0.079
Barrier et al. (2006) [21]		30	30 Affymetrix probe sets	0.095
Lin et al. (2007) [22]	New Zealand data	22	15 Affymetrix probe sets	0.006
	German data	13	10 Affymetrix probe sets	0.014
Wang, Barrier and Lin [21–23]	Combined gene set	88	77 Affymetrix probe sets	0.011

Data of previously published prognostic gene expression signatures concerning patients with stages I and II colon cancer were analyzed. The combined gene set comprises all corresponding Affymetrix probe sets including one overlapping gene in the New Zealand and German data set of Lin et al. (PBK). The corresponding probe sets were applied to our data using "global tests" (*p* value)

Since the RNA from these tissue samples was used for gene expression analysis, these cases were designated as "RNA collection" (Table 1).

Patients

"Protein collection" (n=68): Fourteen patients developed relapse (group R) during follow-up (liver metastases in 11 patients, lung metastases in 3 patients, and additional peritoneal carcinosis in 4 patients), 13 (93%) of these patients within 3 years after surgery. The mean time to relapse was 16.5 months (range, 6–33 months). The other 54 patients remained disease free (group DF) for at least 60 months with a mean follow-up of 86 months (range, 60–96 months). Nineteen tumor samples (28%) were shown to be MSI, whereas 49 cases (72%) were designated as MSS (Table 1).

"RNA collection" (n=53, subgroup of "protein collection"): Thirteen patients developed relapse (group R) during follow-up (liver metastases in 10 patients, lung metastases in 3 patients, and additional peritoneal carcinosis in 4 patients), 11 (85%) of these within 3 years of surgery. The mean time to relapse was 16 months (range, 6–33 months). The other 40 patients remained disease free (group DF) for at least 60 months with a mean follow-up of 85 months (range, 60–96 months). Nineteen tumor samples (36%) were shown to be MSI, whereas 34 cases (64%) were designated as MSS (Table 1). Based on these features, the "protein collection" and "RNA collection" can be regarded as very comparable groups with largely overlapping characteristics.

Patients with and without relapse did not differ with respect to the clinical parameters sex, tumor localization, microsatellite status, grading, and age in both collections. Only T-stage showed a significant difference in distribution between the group DF and group R (p=0.015 for protein collection and p=0.001 for RNA collection). Cox regression analysis with clinical parameters in both collections demonstrated the effect of T-stage on the relapse time and slight effects of the parameters tumor localization and microsatellite status.

Protein expression patterns of genes from prognostic gene expression classifiers

Combined published prognostic signatures for prediction of relapse in UICC stage II colon cancer [21–23] comprised a total of 77 genes with only one overlapping gene (PBK) in the New Zealand and Germany data set of Lin et al. [22] (Table 2). We selected six proteins (Table 3) from the 77 signature genes for immunohistochemical validation by analyzing distribution, intensity, and localization of the staining in FFPE sections of 68 cases of UICC II colon carcinoma. Overall, the concordance between the RNA and protein signal of all analyzed genes and proteins was high, resulting in a strong statistical association (Fig. 1).

Cadherin 17 (CDH17) and tumor necrosis factor receptor superfamily member 11a (TNFRSF11A) were both

Table 3 Prognostic proteins

Selected proteins for validation of published prognostic gene expression signatures

Protein symbol	Protein full name	Dataset	Function
CDH17	Cadherin 17	Wang et al. [23]	Cell adhesion; potential role in lymph node metastasis and progression of human CRC [41]
TNFRSF11A	Tumor necrosis factor receptor superfamily, member 11a	Lin et al. [22]	Member of TNF receptor superfamily; activation of NFkB [42]
LAT	Linker for activation of T cells	Wang et al. [23]	Transmembrane protein; recruits signaling molecules near the site of TCR engagement [43]
EMR3	Egf-like module containing, mucin-like, hormone receptor-like 3	Wang et al. [23]	Transmembrane receptor family; probably involved in myeloid interactions during immune and inflammatory responses; expressed predominantly by cells of the immune system [44]
CA2	Carbonic anhydrase II	Lin et al. [22]	Catalyzes reversible hydration of carbon dioxide; downregulated in neoplastic colorectal mucosa compared to normal colorectal mucosa [45]
PBK	PDZ binding kinase	Lin et al. [22]	Mitotic kinase; upregulated in testis and highly proliferating normal (placental cells, lymphoid cells) and malignant cells (breast cancer, Burkitt lymphoma) [46, 47]

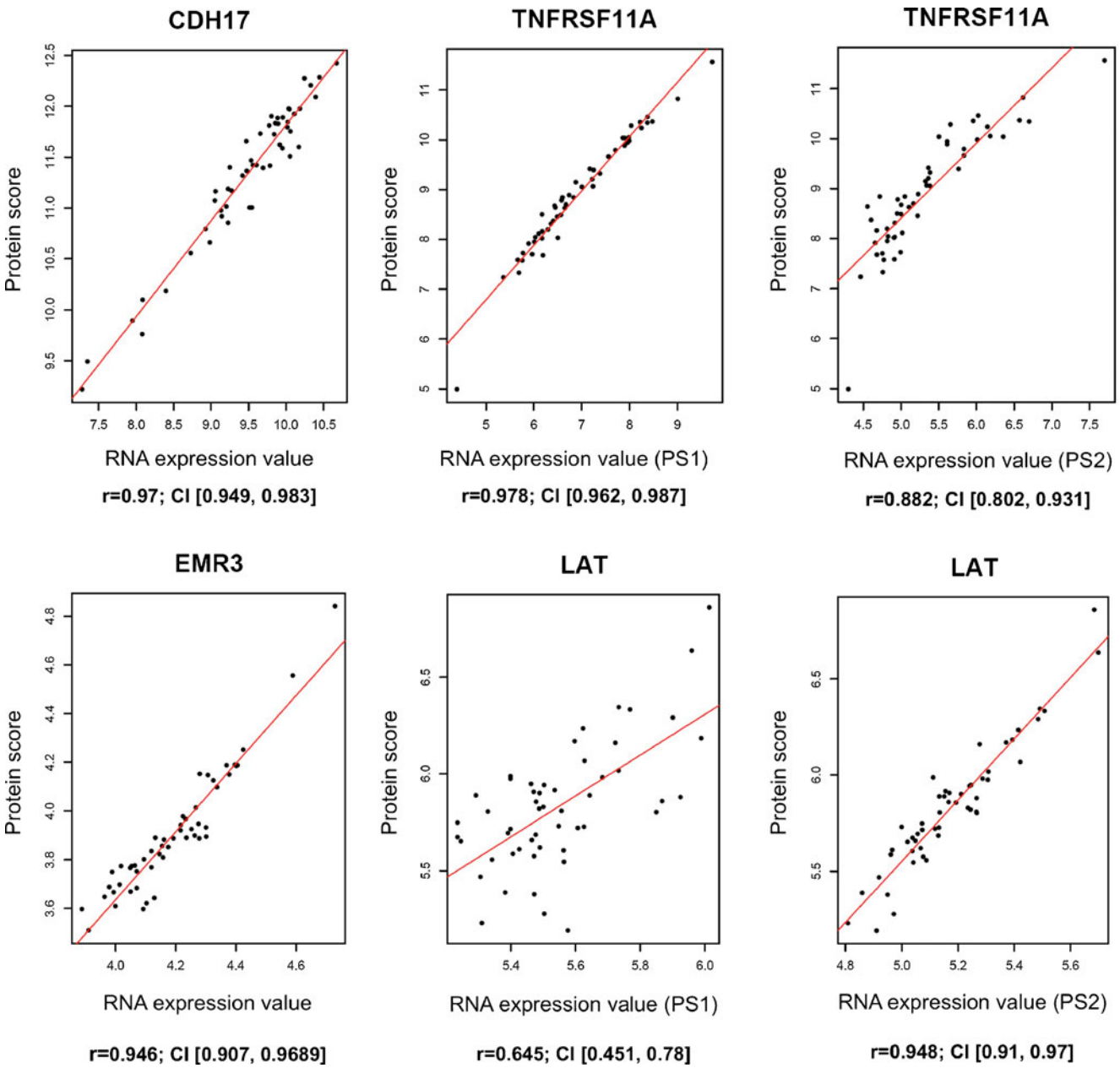

Fig. 1 Scatter plots. Correlation of expression data of selected proteins with expression in tumor tissue and corresponding RNA probe sets (scatter plot; regression line in *red*). Scores of protein expression were given on *y-axis* and gene expression values (quantile normalized log2 signal intensity) on the *x-axis* (see "Methods"). TNFRSF11A and LAT were represented by two probe sets on the array (PS1 and PS2) and separately correlated with protein expression resulting in two scatter plots, respectively

expressed on the membrane of the tumor cells. The staining intensity and the percentage of positive tumor cells for both markers varied between the cases as well as within the same case (Fig. 2a–f). Furthermore, expression of CDH17 was lower in dedifferentiated tumors than in well-differentiated tumors. Antibodies against linker for activation of T cells (LAT) and egf-like module containing, mucin-like, hormone receptor-like 3 (EMR3) showed membranous staining of cells of the tumor stroma, namely T cells and granulocytes and monocytes, respectively (Fig. 2g, h). Expression of carbonic anhydrase II (CA2) was found only in the cytoplasm and nuclei of cells of the normal colonic mucosa (Fig. 2i). Accordingly, no scoring was applied for this marker. Repeated staining of PBK with different commercially available antibodies showed inconsistent results regarding intensity, distribution, and localization and was thus not further exploited.

Correlation of the protein expression scores of CDH17, TNFRSF11, EMR3, and LAT with the relapse status of all patients showed no significant association for each of the

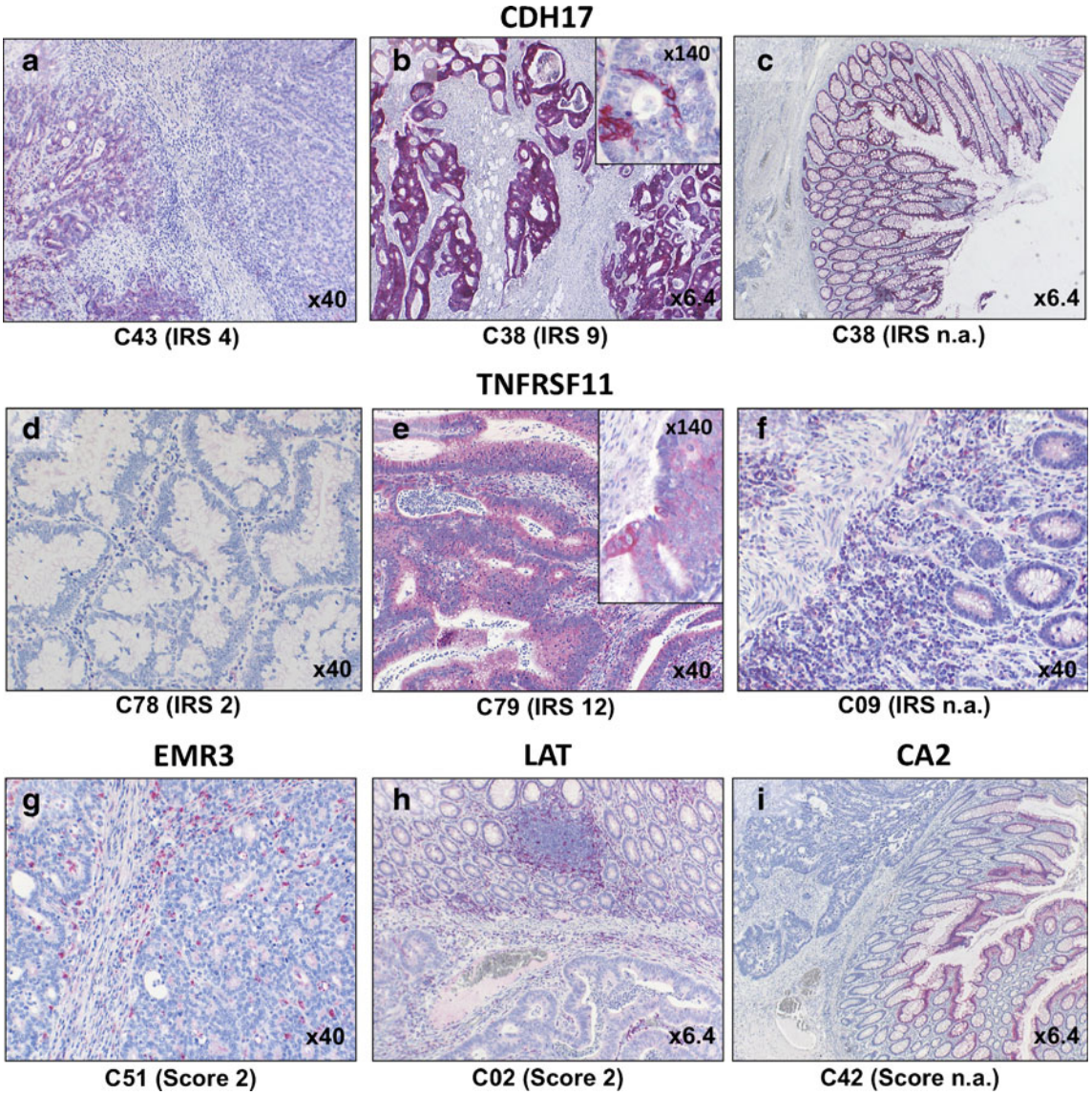

Fig. 2 IHC staining. Immunohistochemical stainings of paraffin sections from colon carcinoma cases for CDH17 (**a**–**c**), TNFRSF11A (**d**–**f**), EMR3 (**g**), LAT (**h**), and CA2 (**i**). Tumor cells show strong staining heterogeneity within the same tumor as well as between tumors when stained for CDH17 (**a**, **b**) and TNFRSF11A (**d**, **e**). CDH17 is additionally expressed on cells of the normal colonic mucosa (**c**), whereas TNFRSF11A is also expressed by tumor-infiltrating lymphocytes (**f**). EMR3, LAT, and CA2 are not expressed by the colon carcinoma tumor cells. EMR3 (**g**) is exclusively produced by stromal macrophages, granulocytes, and monocytes whereas LAT (**h**) is restricted to tumor-infiltrating T cells. CA2 (**i**) is only found on cells of the normal colonic mucosa. Images were taken with an Olympus AH-2 microscope (Olympus, Tokyo, Japan) equipped with a Hitachi HV-F22 CCD camera (Hitachi Europe, Maidenhead, Great Britain) using Diskus v4.70 software (Hilgers, Königswinter, Germany)

four markers with patient prognosis (i.e., disease-free versus relapse) in Cox regression (Fig. 3a–d).

Development of classification gene expression signatures to identify patients with recurrent disease

In order to identify the patients with recurrent colon carcinoma in our cohort, we first used a binary classification approach comparing the gene expression data of all relapsed patients with those patients who remained disease-free up to 5 years after surgery. Unexpectedly, all UICC II patients irrespective of their clinical outcome were classified into the good prognosis group (DF). Analyses of the subgroup of microsatellite stable (MSS) patients also failed to correctly predict the risk of relapse by gene expression from our data. In a further approach, the time to relapse was used as a continuous outcome variable employing probe sets selected from our own experiments as well as the pooled probe sets from signatures that had been published to be predictive of a poor prognosis (relapse). Again, no

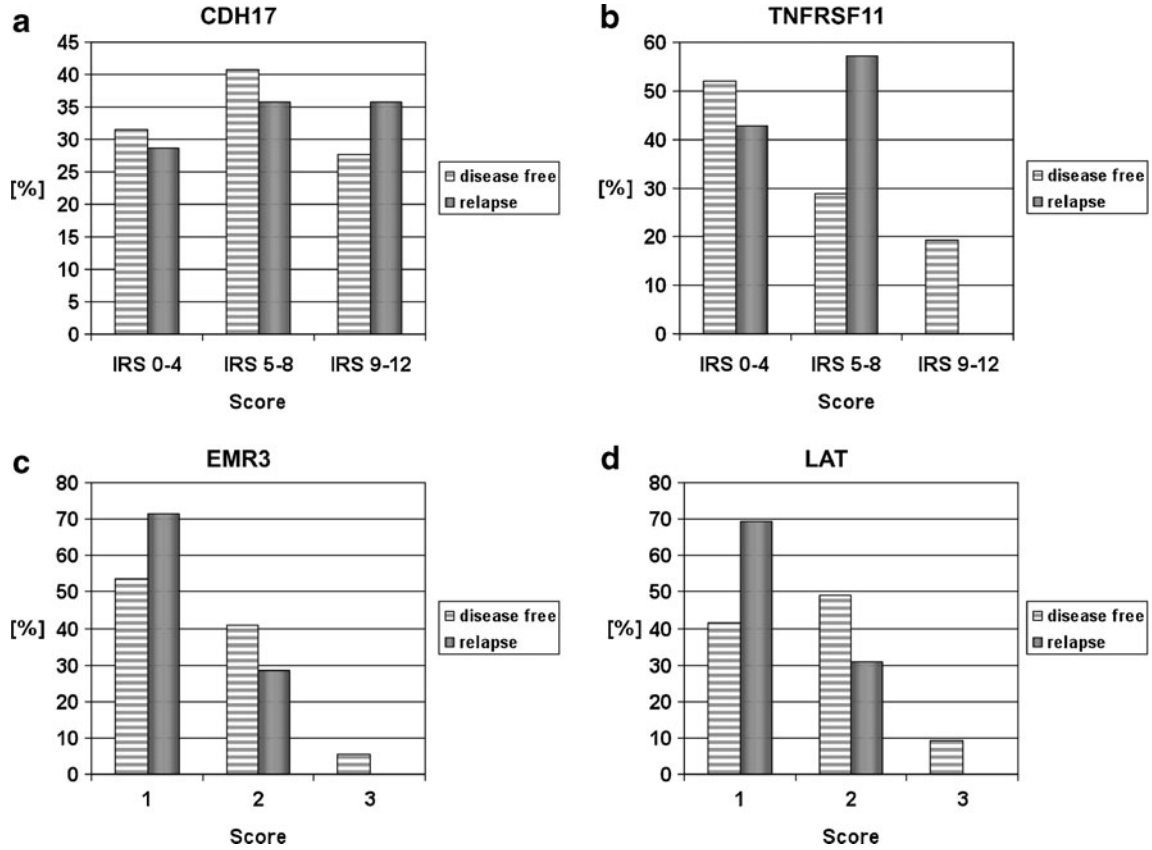

Fig. 3 IHC score. Results of semiquantitative immunohistochemical scores for CDH17 (**a**) and TNFRSF11A (**b**) (immunoreactivity score) and for EMR3 (**c**) and LAT (**d**) (scored by the number of positive cells (1=low number, 2=moderate number, 3=high number of cells)). Percentage of patients with either IRS 0–4, 5–8, and 9–12 (CDH17 and TNFRSF11A) or score 1–3 (EMR3 and LAT)

gene expression signature predictive for the patients' outcome could be constructed, and furthermore, also the published prognosticators were not valid in our data set.

When the different published prognostic signatures for prediction of relapse in UICC stage II colon cancer [21–23] were applied to our gene expression data set, only the corresponding probe sets from the signatures of Lin et al. [22] were able to separate the relapse and disease-free group using "global test" (Table 2). By penalized Cox regression, three genes with a high influence on separation in the "global test," i.e., PBK, CXCL11, and CA2, were identified in the Lin et al. signature from the German data [22]. All of these markers showed higher expression in patients with disease-free follow-up (Fig. 4a–e). The application of support vector machine and leave-one-out cross-validation to construct a classification rule from our data resulted in a positive prediction in 75.5% (when the probe sets from the New Zealand signature were used to build the classifier) and 64.2% (when the probe sets from the German data were used to build the classifier) of the cases. However, both classifiers did not reach significance when testing that the correct classification rate is different from 0.5 (p=0.11; p=0.79 respectively).

Discussion

The introduction of large-scale gene expression analyses enabled molecular classification of patient subgroups even within the same tumor entity by the generation of gene expression signatures. Furthermore, these tumor signatures have shown to be of prognostic value for many types of solid cancer [2, 6–8, 12–15, 17, 29–32]. For stage UICC II colon cancer, four prognostic molecular signatures have independently been reported up to date [21–23]. These signatures comprise 19 to 30 genes and were suggested to identify patients with localized colon cancer at a significantly elevated predicted risk of relapse and to stratify patients to receive adjuvant therapy, similar to stage UICC III colon cancer, with an accuracy of 76.3%–84%.

In spite of these encouraging results, there is currently no clinical risk stratification or adjustment of treatment modalities according to this molecular subgrouping [20]. This may be due to several reasons: (a) for clinical application a signature needs a prognostic sensitivity of at least 85% and a prognostic specificity of at least 80% when the prevalence of high risk patients is not smaller than 10%, (b) the acquisition of gene expression data requires a

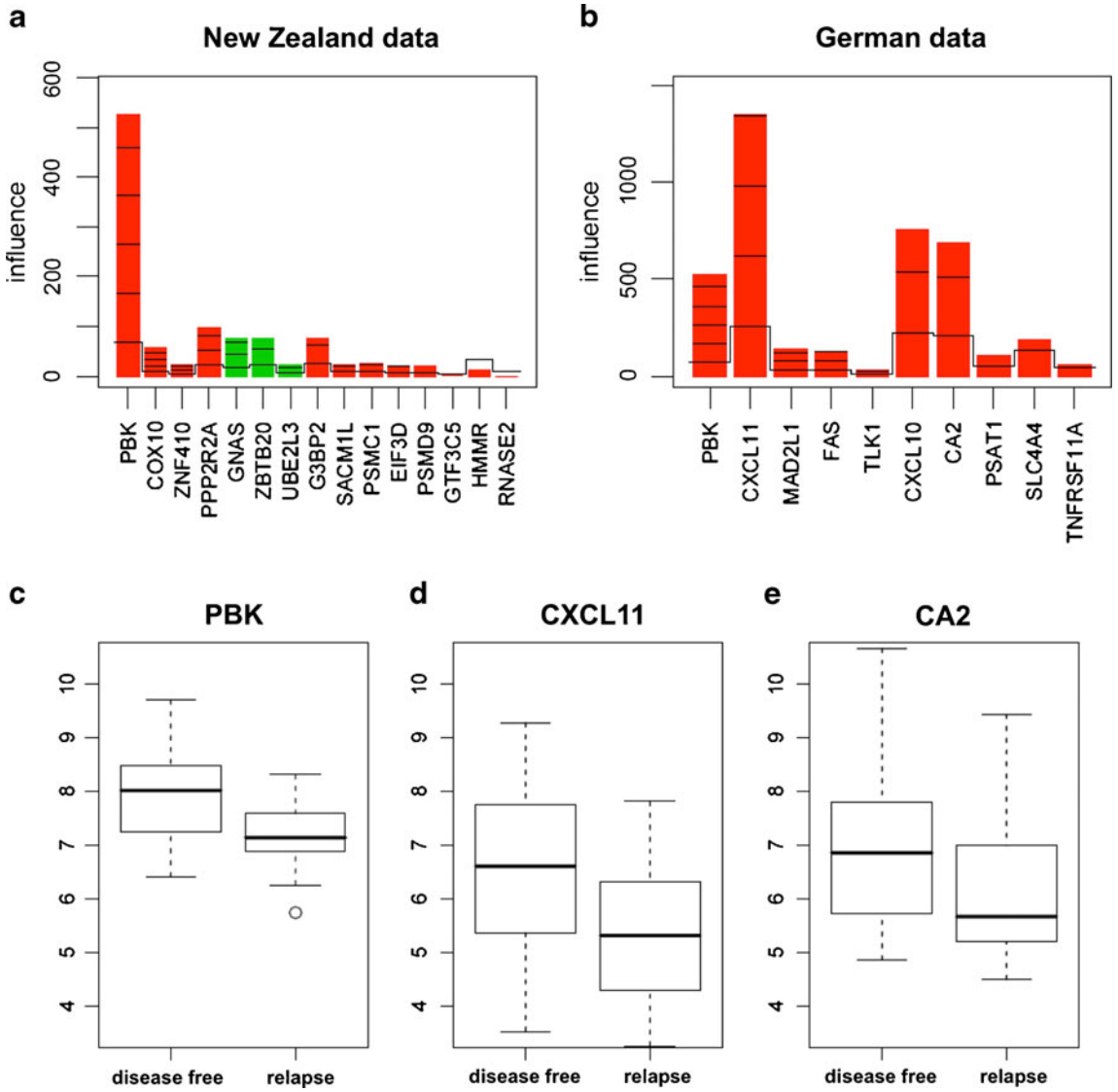

Fig. 4 Gene plots. Gene plots for the probe sets of the signatures of Lin et al. [22], derived from New Zealand data (**a**) and German data (**b**). The plots correspond to "global tests" for differential expression between "relapse within 5 years" and "disease-free" in the data set of UICC stage II patients. Genes with higher expression values in patients without relapse are colored in *red*, and those with higher expression in patients with relapse are colored in green. The *bar* height indicates the influence of each gene on the "global test" statistic. A reference line for each bar gives the expected height of the bar under the null hypothesis that the gene is not associated with the response. Marks indicate with how many standard deviations the bar exceeds the reference line. **c**–**e**: Box plots of normalized expression values for the three genes (PBK, CXCL11, CA2) most associated with patient prognosis. PBK is the only overlapping gene in the New Zealand and German data set of Lin et al., and it is one of the three genes mostly associated with patient prognosis in both gene signatures

molecular and bioinformatic setting, and (c) gene expression analysis requires unfixed and fresh frozen tissue specimens with a sufficient percentage of tumor cells. Each of these reasons is able to prevent the broad introduction of prognostic gene expression signatures into patients' care. Therefore, alternative methods are highly desirable which are applicable to established routine diagnostic procedures such as IHC for the detection of protein expression in tissue sections. IHC is applicable to formalin-fixed and paraffin-embedded tissue samples and it can be standardized for the comparable read-out of the results.

It should be pointed out that none of the RNA-based classifiers for UICC II colon cancer has been validated at the protein level so far. Therefore, it was the primary goal of this study to translate published gene expression classifiers into a prognostic protein signature and to test this signature on primary tumor specimens obtained from UICC II colon cancer patients. For this purpose, we chose six genes (CDH17, LAT, CA2, EMR3, TNFRSF11A, and PBK) from all published gene expression signatures [22, 23, 33] particularly with regard to their involvement in (colon-) carcinogenesis and a positive correlation of protein

and RNA expression. For the quantification of the IHC results, a scoring system considering the staining intensity and the proportion of labeled tumor cells or tumor-infiltrating cells, respectively, was applied. Despite the distinguishable staining patterns of CDH17 and TNFRSF11A within the colon cancer cases tested, there was no correlation of the expression of these two proteins with clinical outcome. The same holds true for EMR3 and LAT that were found to be expressed mainly by reactive lymphoid cells, which were unable to discriminate patients with good and poor prognosis. Accordingly, the prognostic power of the protein data was too weak to identify patients with risk of relapse within our collective, although there was a high degree of correlation between gene expression (RNA) and immunohistochemistry for the selected genes. Furthermore, no statistically significant prediction of the clinical outcome was possible regardless of the combination of protein markers used.

Due to the fact that the published gene expression classifiers could not be translated into a prognostic protein signature, we were interested to validate published gene expression signatures in our own case collection. To this end, Affymetrix HG-U133 Plus 2.0 GeneChips were hybridized with RNA derived from 53 ("RNA collection") of the 68 cases used for IHC analyses ("protein collection").

Our gene expression data confirm that differential gene expression and a molecular subgrouping of relapsed and disease-free patients are also principally possible in our case collection. However, all efforts to derive a statistically stable signature, which allows a prognosis of the clinical course, failed. Despite intensive data mining employing various sophisticated bio-mathematical methods, no gene expression signature could be identified, which enables a reliable and statistically significant prediction in our data set. Moreover, the application of published gene expression classifiers to our data was unable to reproduce previously described prediction of clinical outcome. Since we used enriched tumor cell preparations (80–90%) for our gene expression analysis similar to the other studies in this field [21–23], the inability to reproduce published prognosticators is not due to contaminating non-malignant cells. Furthermore, a bias in our "RNA collection" could be ruled out since these 53 cases displayed the same typical clinical characteristics as the 68 cases of the "protein collection." Furthermore, microsatellite status and age had no significant influence on the results as demonstrated by subgroup analyses.

The failure to detect prognostic gene expression signatures in our cases is also not due to an insufficient validity of the data since a consistent and tremendous expression difference was detectable between normal colonic mucosa cells and colon cancer cells as shown by others [33–35] (data not shown). It is more likely that the gene expression variance within the two experimental groups is too high for significant separation, although we tried to reduce potential heterogeneity by subgroup analyses of MSS patients only.

The discrepancy between our own and published data to identify a prognostic signature in stage UICC II colon cancer patients points to the fact that the available molecular prognosticators are not yet generally applicable. This observation was not only made in the case of stage UICC II colon cancers but for many, if not most, other molecular signatures [36]. Many different reasons appear to contribute to this finding such as the proportion of truly differentially expressed genes, the distribution of the true differences, measurement variability, and sample size [19] as well as statistical approach [37]. To benefit from this promising technology, large studies with appropriate clinical design, adjustment for known predictors, and proper validation are needed [18, 38].

Conclusions

In consequence, although gene expression data can be regarded as very reliable [39], their interpretation and general application to independent case collections and/or individual patients are not yet established. Our protein and gene expression analyses do currently not support application of molecular classifiers for prediction of clinical outcome in routine diagnostics as a basis for patient-orientated therapy in stage UICC II colon cancer. The low prediction rate of published gene signatures hampers the implementation of molecular prognosticators for individualized therapy in stage UICC II colon cancer calling for further studies to develop signatures applicable in patient care.

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