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ORIGINAL ARTICLE

Protein levels of clusterin and glutathione synthetase in platelets allow for early detection of colorectal cancer

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Abstract Colorectal cancer (CRC) is one of the most frequent malignancies in the Western world. Early tumor detection and intervention are important determinants on CRC patient survival. During early tumor proliferation, dissemination and angiogenesis, platelets store and segregate proteins actively and selectively. Hence, the platelet proteome is a potential source of biomarkers denoting early malignancy. By comparing protein profiles of platelets between healthy volunteers ($n = 12$) and patients with early- ($n = 7$) and late-stage ($n = 5$) CRCs using multiplex fluorescence two-dimensional gel electrophoresis (2D-DIGE), we aimed at identifying differentially regulated proteins within platelets. By inter-group comparisons, 94 differentially expressed protein spots were detected ($p < 0.05$) between healthy controls and patients with early- and late-stage CRCs and revealed distinct separations between all three groups in principal component analyses. 54 proteins of interest were identified by mass spectrometry and resulted in high-ranked Ingenuity Pathway Analysis networks associated with *Cellular function and maintenance*, *Cellular assembly and organization, Developmental disorder* and *Organismal injury and abnormalities* ($p < 0.0001$ to $p = 0.0495$). Target proteins were validated by multiplex fluorescence-based Western blot analyses using an additional, independent cohort of platelet protein samples [healthy controls ($n = 15$), early-stage CRCs ($n = 15$), late-stage CRCs ($n = 15$)]. Two proteins—clusterin and glutathione synthetase (GSH-S)—featured high impact and were subsequently validated in this independent clinical cohort distinguishing healthy controls from patients with early- and late-stage CRCs. Thus, the potential of clusterin and GSH-S as platelet biomarkers for early detection of CRC could improve existing screening modalities in clinical application and should be confirmed in a prospective multicenter trial.

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Keywords Clusterin · Colorectal cancer · Early detection · Glutathione synthetase · Platelet protein

Abbreviations

2D-DIGE	Two-dimensional multiplex fluorescence gel electrophoresis
BHT	Butylated hydroxytoluene
CLU	Clusterin
CRC	Colorectal cancer
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
EMT	Epithelial-to-mesenchymal transition
FC	Fold change
GSH-S	Glutathione synthetase
IEF	Isoelectric focusing
IPA	Ingenuity Pathway Analysis
IPG	Immobilized pH gradient
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time of flight mass spectrometry
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PMSF	Phenylmethylsulfonyl fluoride
PTMs	Posttranslational modifications
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
UICC	International Union Against Cancer

Introduction

Various cells of tissues and organs release or secrete proteins into the blood. Accordingly, the quantitative protein composition of blood as well as its subcompartments and cellular components provide crucial information reflecting the current physiological or pathological status of an organism [1–4]. In this context, tumor cells directly interact with all constituents of the hematological system, including platelets [5, 6]. Besides their crucial role in hemostasis, circulating platelets are involved in primary tumor proliferation, angiogenesis, cancer cell protection, promotion of epithelial-to-mesenchymal transition (EMT) and hematogenous metastasis [6–9]. Hereby, the consequence of tumor cell-induced platelet activation and subsequent aggregation through direct and indirect mechanisms could lead to cancer spread which is strongly associated with a hypercoagulable state found in most cancer patients [10, 11].

Platelets are cell fragments without nucleus derived from megakaryocytes and limited in protein synthesis. In between healthy individuals, the platelet proteome is highly similar and comprises >5000 proteins [12]. Turnover time of platelets is as short as 5–7 days [7]. Therefore, the composition of the platelet protein content reflects the current physiological state and is highly dynamic during pathophysiological processes. In line, platelet disorders and dysfunctions correlate with alterations in the platelet proteome and extensive dynamic differences regarding posttranslational modifications (PTMs) [12, 13]. Increased expression levels of platelet adhesion receptors (e.g., P-selectin, CD62, CD63) have been noticed as common findings in blood of cancer patients and indicate an activated status of platelets [14, 15]. Furthermore, the concentration of β-thromboglobulin, a marker of platelet activation, is significantly increased in many carcinomas (e.g., cancer of the breast, lung, gastric, prostate, and colon) with particular impact on carcinogenesis [5]. By means of three specific types of secretory granules (dense granules, α-granules and lysosomal granules), platelets store and deliver distinct content which is released upon activation and aggregation processes. [16–18]. It has been demonstrated that tumor cells rely on platelet-derived signals outside the primary tumor and that platelets are widely recognized as dynamic reservoirs of tumorigenic growth factors as well as proangiogenic and prometastatic proteins. In vivo (pre)activation of platelets with impact on (patho)physiological platelet protein levels and compositions is described to contribute to modulation of inflammation and carcinogenesis [6, 8, 19–21]. It has been shown that platelets are able to reprogram cancer cells to regulate cyclooxygenase (COX)-2 and prostanoids which modulate cell proliferation and apoptosis mainly in solid tumors and could be used as a therapy targets [22–25].

Platelets potentially act as mediators of colorectal cancer (CRC) which ranks among the most frequent malignancies and is the fourth leading cause of cancer-related death worldwide [26, 27]. Diagnosis of CRC occurs late, since symptoms rarely appear at first, and prognosis is highly dependent on tumor staging. Thus, early tumor detection and curative treatment interventions are important determinants on CRC patient survival. Although 5-year cancer-free survival exceeds 90% for International Union Against Cancer (UICC) stage I carcinomas, this percentage is reduced to 63% in UICC III and <5% in UICC IV tumors [28, 29]. Therefore, innovative tools for early CRC detection are of high clinical importance. With the knowledge that platelets promote tumor growth and metastasis [30], platelet proteins could thus serve as minimal-invasive screening markers for CRC diagnosis. Nevertheless, platelet biomarker analysis is still challenging due to potential ex vivo activation during platelet isolation steps which has a serious impact on (patho)physiological protein levels and protein composition [12, 31].

In this study, we hypothesize that platelet protein profiles of patients with early- and late-stage CRCs differ from those of healthy donors. To analyze the intact platelet proteome in terms of different protein levels between groups,

two-dimensional multiplex fluorescence gel electrophoresis (2D-DIGE) with subsequent mass spectrometry (MS) was applied. Next to the benefit of evaluating PTMs and hereby biological significant processes, the visualization of thousands of—mainly hydrophilic—protein spots by 2-D DIGE can be understood as a proteomic blueprint of a given sample including quality assurance [32]. Identified target proteins were characterized by Ingenuity Pathway Analysis (IPA) and validated using a quantitative, high-sensitivity multiplex fluorescence-based Western blot approach in an independent patient cohort of platelet proteins. For overall study design, please see Supplementary Fig. S1.

Materials and methods

Patient sample collections

This study was approved by the local Ethics Committee of the University of Lübeck (#07-124). Blood samples of CRC patients and healthy volunteers were processed after informed consent and stored at the Interdisciplinary Centrum for Biobanking-Lübeck (ICB-L). Platelets were obtained pre-therapeutically. Tumors classified as CRC were divided with regard to their pathological disease stage into early-stage (UICC I and II) and late-stage (UICC III and IV). Platelets from patients who underwent an unobtrusive colonoscopy without any history of malignancy or platelet disorders were collected and used as healthy controls.

For 2D-DIGE protein profiling, CRC patient population consisted of 12 cases with seven early-stage CRCs (UICC I and II) and five late-stage CRCs (UICC III and IV) (Table 1; Supplementary Table S1). Platelet samples from 12 healthy individuals were used as controls.

Protein validation was performed by multiplex fluorescence-based Western blot analyses using an independent clinical cohort of platelet samples from 15 healthy volunteers and 30 CRC patients (Table 2; Supplementary Table S2). The latter was characterized by 15 early-stage CRC (UICC I and II) and 15 late-stage CRC (UICC III and IV) patients.

Platelet isolation

The ethylenediaminetetraacetate (EDTA) anticoagulated venous blood was centrifuged at 200×*g* for 20 min at room temperature. The upper two-thirds of the platelet-rich plasma supernatant was carefully removed by aspiration, diluted 1:3 with 1× Dulbecco's phosphate-buffered saline (PBS) containing neither Ca^{2+} nor Mg^{2+}, and centrifuged at 800×*g* for 10 min at room temperature. Subsequently, the platelet pellet was resuspended in 1 ml 1× PBS and centrifuged at 800×*g* for 10 min at room temperature. After removing the supernatant, the platelet pellet was dissolved in lysis buffer

Table 1 Patient cohort for 2D-DIGE protein profiling of platelets

	Value	Healthy volunteers	Early-stage CRC	Late-stage CRC	Total CRC
Total		12	7	5	12
Sex	Male	3 (25%)	3 (43%)	3 (60%)	6 (50%)
	Female	9 (75%)	4 (57%)	2 (40%)	6 (50%)
Age at diagnosis	Median (years)	63.4	68.4	72.8	68.9
	Range (years)	39.5–87.1	52.5–81.6	68.6–81.2	52.5–81.6
UICC-stage	I		2 (29%)		2 (17%)
	II		5 (71%)		5 (42%)
	III			4 (80%)	4 (33%)
	IV			1 (20%)	1 (8%)

Table 2 Patient cohort for multiplex fluorescence-based Western blot platelet validation

	Value	Healthy volunteers	Early-stage CRC	Late-stage CRC	Total CRC
Total		15	15	15	30
Sex	Male	6 (40%)	7 (47%)	7 (47%)	14 (47%)
	Female	9 (60%)	8 (53%)	8 (53%)	16 (53%)
Age at diagnosis	Median (years)	57.3	74.4	62.1	69.7
	Range (years)	32.7–79.6	55.1–83.1	42.9–91.1	42.9–91.1
UICC-stage	I		3 (20%)		3 (10%)
	II		12 (80%)		12 (40%)
	III			9 (60%)	9 (30%)
	IV			6 (40%)	6 (20%)

[9 M urea, 65 mM dithiothreitol (DTT), 1 mM EDTA, 0.5% (v/v) NP-40, 1.5% (w/v) CHAPS, 35 mM NaOH, 0.0001% (w/v) phenylmethylsulfonyl fluoride (PMSF), 0.01% (w/v) benzamidine, 0.01% (w/v) butylated hydroxytoluene (BHT)] and stored at −80 °C until processing 2D-DIGE and Western blot analyses, respectively.

Potential ex vivo activation of the platelets during sample handling and in vitro isolation was excluded as follows: after platelet isolation, one part of the sample was artificially activated by adding 5 µg/ml collagen, 0.5 U/ml thrombin and 13.8 mmol/l $CaCl_2$. Compared to the non-affected isolate, platelet activation and aggregation was visible as white aggregate after addition of agonists (Supplementary Fig. S2).

Protein precipitation and quantification

Proteins of each platelet sample were precipitated with the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad Laboratories, USA) and diluted in 2D-DIGE sample buffer [30 mM TRIS, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS]. Total protein concentration was subsequently determined in quadruples using the fluorescence-based EZQ™ Protein Quantitation Kit (Life Technologies, USA). Fluorescence visualization was carried out with the Typhoon™ FLA 9000 laser scanner (GE Healthcare). Densitometric analysis was performed using the ImageQuant™ TL software (GE Healthcare).

Two-dimensional multiplex fluorescence gel electrophoresis (2D-DIGE)

A total of 50 µg of each platelet protein sample and a pooled internal standard examining gel-to-gel variation was minimally labeled with the fluorescence-based Refraction-2D™ Labeling Kit (NH DyeAGNOSTIC, Germany) according to the manufacture's protocol. 150 µg protein per gel (2 × 50 µg sample plus 50 µg internal standard) were diluted with rehydration sample buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) ampholytes (pH 4–7, SERVA Electrophoresis, Germany) and a trace of bromophenol blue] to a final volume of 450 µl and applied to immobilized pH gradient (IPG) gel strips with a pH range 4–7 (Immobiline DryStrip pH 4–7, 24 cm, linear, GE Healthcare) by means of an active sample in-gel rehydration approach under gentle voltage (50 V, 6 h). Isoelectric focusing (IEF) was carried out in a Protean® i12™ IEF cell (Bio-Rad Laboratories, USA) at 20 °C reaching approximately 57,700 Vh. After IEF, the IPG strips were immediately equilibrated for 2 × 15 min in a premade buffer system containing Tris–Tricine/SDS (pH 6.9) (Buffer Kit for 2D HPE™ Gels, SERVA Electrophoresis). To reduce S–S bonds and alkylate free thiols, DTT [2% (w/v)] was included to the buffer in the first and iodoacetamide [2.5% (w/v)] in the second equilibration step. The horizontal second dimension (HPE™ FlatTop Tower, SERVA Electrophoresis) was carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on precast plastic-backed 12.5% acrylamide gels (2DHPE™ Large Gel NF 12.5% Kit, 0.65 × 200 × 255 mm, SERVA Electrophoresis). Electrophoresis was accomplished with an increasing gradient at 12 °C reaching approximately 3400 Vh.

Image acquisition and statistical analysis

Gel image acquisition was performed immediately after SDS-PAGE by using a Typhoon™ FLA 9000 laser scanner (GE Healthcare). Subsequently, protein spots were analyzed using the software Progenesis SameSpots® (Nonlinear Dynamics, UK, v4.1). Matching results of the fully automated gel-to-gel algorithm provided by the software were manually controlled in order to avoid and correct automated matching errors. For long-term storage, 2D-DIGE gels were fixed [40% (v/v) ethanol, 50 mM citric acid, 10% (v/v) acetic acid] and conserved [3% (v/v) glycerol].

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)

For matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) analysis, significant different protein spots ($p \leq 0.05$) were cut from fixed 2D-DIGE gels into 96-well plates (DigestPro 96 well reaction plate, INTAVIS Bioanalytical Instruments, Germany) using the automated Ettan Spot Picker (GE Healthcare). Peptides were extracted as described [33]. Subsequently, samples were analyzed in an Ultraflex MALDI-TOF/TOF mass spectrometer (BRUKER Daltonics). Acquired mass spectra were automatically calibrated and annotated using Compass 1.3 for flex software (BRUKER Daltonics). For protein identification, results from each individual protein spot were used to search a human subset in Swiss-Prot (Sprot_57.8, 20,401 protein entries) non-redundant database by means of Mascot search engine (Matrix Science Ltd., UK, v2.2) in consideration of the following settings: (1) enzyme "trypsin"; (2) species "human"; (3) fixed modifications "carbamidomethyl"; (4) optional modifications "methionine oxidation" and (5) missed cleavages "1". Mass tolerance was set to 50 ppm. Using these settings, a Mascot score >70 was taken as significant ($p \leq 0.01$).

Ingenuity Pathway Analysis (IPA)

Ingenuity® Pathway Analysis™ (IPA®) software (QIAGEN Silicon Valley, USA) was used to investigate possible interactions between all identified proteins in distinct group comparisons. Interactive pathways were generated to observe

relations among differentially expressed proteins. Networks with a score ≥5 were considered significant.

Multiplex fluorescence-based Western blot analysis

Multiplex fluorescence-based Western blot analyses were performed using a modified protocol [34]. Platelet protein samples and a pooled internal standard of all samples to normalize gel-to-gel variations were minimally labeled with the high-performance fluorescent G-Dye300 (NH DyeAGNOSTIC, Germany) according to the manufacture's protocol. Protein samples and the internal standard were diluted with 1× Tris-buffered saline (TBS, pH 7.4) and 1:2 in 2× Laemmli sample buffer (Bio-Rad Laboratories) to a final volume of 10 µl before loading on precast 4–15% polyacrylamide gels (4–15% Criterion™ TGX™ Protein Gel, 26 well, 15 µl, 13.3 × 8.7 cm, Bio-Rad Laboratories). Electrophoresis was carried out in 1× Tris/glycine/SDS running buffer (Bio-Rad Laboratories) at constant 200 V for 35 min in a Criterion™ Vertical Electrophoresis Cell (Bio-Rad Laboratories). A fluorescent protein standard (Precision Plus Protein™ WesternC™ Standard, Bio-Rad Laboratories) was used to track protein migration. After SDS-PAGE, separated proteins were electroblotted with 25 V and 1.0 A within 30 min onto a PVDF membrane (Immobilon®-FL PVDF, 0.45 µm, Merck Millipore) using a Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories). The membrane was blocked at room temperature for 1 h with 2% Amersham ECL Prime Blocking Agent (GE Healthcare), dissolved in 1× TBS with 0.1% Tween-20 (pH 7.6, Cell Signaling, USA) and incubated with primary antibodies against clusterin (rabbit monoclonal antibody, clone EPR2911, TA307501, OriGene), cofilin-1 (mouse monoclonal antibody, clone E-8, sc-376476, Santa Cruz Biotechnology, Inc., USA) and glutathione synthetase (rabbit polyclonal antibody, clone H-300, sc-28966, Santa Cruz Biotechnology) in 2% blocking buffer at 4 °C overnight. For each primary antibody, a suitable condition regarding antibody dilution and protein sample concentration was determined in preliminary experiments (clusterin 1:1000/5.0 µg; cofilin-1 1:500/2.5 µg; glutathione synthetase 1:500/6.0 µg). Blots were incubated for 1 h at room temperature with Cy3-conjugated goat-anti-mouse or goat-anti-rabbit secondary antibodies (Amersham ECL™Plex CyDye-Conjugated Antibodies, GE Healthcare) diluted 1:2500 in 2% blocking buffer. Final protein fluorescence visualization was carried out with a Typhoon™ FLA 9000 laser scanner (GE Healthcare). Densitometric analyses of loaded total protein and antibody-targeted protein bands were performed using the ImageQuant™ TL software (GE Healthcare). In order to ensure reproducibility between all protein samples, each specific antibody-targeted protein band (Cy3 channel detection) was first normalized against the loaded total protein (Cy5 channel detection) of the corresponding platelet sample. Afterwards each platelet sample was normalized against the pooled internal standard. The density of a given protein band was measured as the total volume under the three-dimensional peak with background subtraction set to rolling ball for antibody-targeted protein bands.

Statistical analysis

2D-DIGE protein spot expression data were statistically analyzed using Progenesis SameSpots® (Nonlinear Dynamics, UK, v4.1) software. Differences in protein levels and corresponding fold changes (FC) were obtained using following group comparisons:

1. Healthy controls vs. early-stage CRCs vs. late-stage CRCs.
2. Healthy controls vs. CRCs (early-stage and late-stage CRCs).
3. Healthy controls vs. early-stage CRCs.
4. Healthy controls vs. late-stage CRCs.
5. Early-stage CRCs vs. late-stage CRCs.

Student's *t* test (two-group comparison) and 1-way ANOVA (three-group comparison) were used to calculate significant differences in relative abundances of protein spot features. Protein spots with a p value <0.05 were regarded to be significantly different between the groups. Supervised principal component analysis (PCA) of all significant spots was performed to control sample clustering.

For Western blot data, statistical analyses were performed using IBM SPSS Statistics and GraphPad PRISM software. For two-group comparison, Western blot data were calculated using one-tailed Mann–Whitney U test. Comparison between three groups was carried out by Kruskal–Wallis test. A p value <0.05 was considered as significant. Cut-offs for dichotomization into healthy control or early-stage CRC patient were based on maximal sensitivity or specificity values using ROC curve calculation.

Results

Assessment of significant differences in platelet protein profiles between healthy controls and CRC patients by 2D-DIGE

In order to screen for CRC biomarkers, protein profiles were compared between platelets derived from healthy controls (n = 12) and patients with early- (n = 7) and late-stage (n = 5) CRCs using 2D-DIGE (Table 1).

A total of 1178 protein spots within a single gel were detected across all platelet samples with Progenesis SameSpots® software. Regarding two-group comparisons,

overall statistical analyses indicated 94 protein spots with significant changes in their abundances between (1) healthy controls vs. CRC patients (early- and late-stage); (2) healthy controls vs. early-stage CRCs; (3) healthy controls vs. late-stage CRCs and (4) early-stage CRCs vs. late-stage CRCs (Fig. 1). Of those, 50 protein spots were significantly decreased in CRC platelets, whereas 44 protein spots were present at higher levels in CRC compared to healthy control platelets. Supervised PCA of significant spots resulted in distinct group clustering of all two-group comparisons (Fig. 2a, b; Supplementary Fig. S3a, b). Furthermore, 39 differentially expressed protein spots were detected by the three-group comparison (healthy controls vs. early-stage CRCs vs. late-stage CRCs) and revealed distinct separation between the groups in the PCA-based cluster analysis (Supplementary Fig. S3c).

Mass spectrometry and pathway analysis

All 94 spots found to be significantly different were selected for subsequent MALDI-TOF/TOF–MS analysis. 71 (76%) proteoforms representing 54 proteins were unambiguously identified using Mascot database

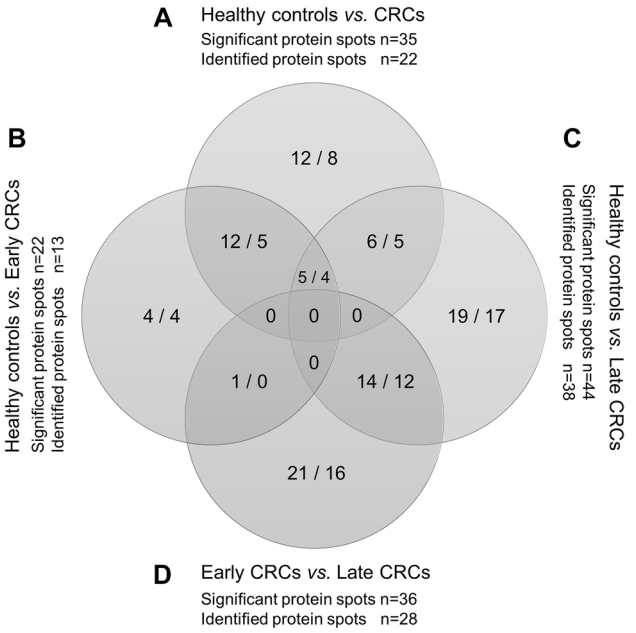

Fig. 1 Venn diagram summarizing distributions and overlaps of 2D-DIGE platelet protein spots between healthy controls and patients with early- and late-stage CRCs. Venn diagram summarizing distributions and overlaps of 94 significant differentially expressed 2D-DIGE protein spots (*t* test, $p < 0.05$) obtained by following two-group comparisons: **a** healthy controls ($n = 12$) vs. CRCs (early-stage and late-stage, $n = 12$); **b** healthy controls ($n = 12$) vs. early CRCs ($n = 7$); **c** healthy controls ($n = 12$) vs. late CRCs ($n = 5$), and **d** early CRCs ($n = 7$) vs. late CRC ($n = 5$). Distribution of corresponding 71 protein spot identifications is represented by the numerals after slash

(Supplementary Table S3). IPA was used in order to provide functional networks and canonical pathways reflecting the physiological relationships between these 54 protein identities. Concerning the comparison of healthy controls vs. CRC patients, 14 out of 15 identified proteins were recognized by the Ingenuity knowledge database, namely AHCY, B2M, CLU, FAM49A, FDPS, FGB, GSS, HDHD2, ITGA6, MLEC, PDIA6, TLN1, UNC45A, VCL (Fig. 3a; Supplementary Table S4). One network reached the level of significance with a score of 40 and was associated with *Developmental disorder*, *Hematological disease* and *Hereditary disorder*. Focusing on healthy controls vs. early-stage CRCs comparison, one top ranked network linked to *Organismal injury and abnormalities*, *Reproductive system disease* and *Immunological disease* was ascertained with a score of 41 (Fig. 3b; Supplementary Table S4). This included 14 differentially expressed and identified proteins: ACTB, CASP3, CLU, FAM49A, FDPS, FGB, GSS, HDHD2, HSP90B1, KRT5, LCP1, MLEC, PDIA6, TLN1. Both networks exhibited polyubiquitin-C (UBC) as central node and an overlap of nine proteins (CLU, FAM49A, FDPS, FGB, GSS, HDHD2, MLEC, PDIA6, TLN1). All proteins are involved in cancer-associated functions such as *Cell-to-cell signaling and interaction*, *Cellular assembly and organization*, *Cellular movement*, *Cell death* and *Survival and cellular growth and proliferation* ($p < 0.0001$ to $p = 0.0495$; Supplementary Table S4). All targets were evaluated for their biological function by an individual IPA database search. Cancer relevant candidates were subsequently subjected to a Pubmed-based literature search using the following term: (”x”[tiab]) AND (cancer[tiab] OR tumor[tiab] OR carcinoma[tiab]) AND Humans[Mesh] AND English[lang]), whereby “x” stands for each candidate after IPA search. Bibliographies of the articles discovered were additionally checked for relevant citations.

Clusterin, glutathione synthetase (GSH-S) and cofilin-1 were selected accordingly as protein targets for subsequent Western blot validation. Selection was based on biological function, 2D-DIGE protein levels, *p* values and fold changes (FC) of clusterin and GSH-S in healthy controls vs. CRC patients (early- and late-stage) and in healthy controls vs. early-stage CRCs comparisons (Supplementary Fig. S4a, b), as well as on 2D-DIGE data of clusterin and cofilin-1 in healthy controls vs. late-stage CRCs and three-group comparison (healthy controls vs. early-stage CRCs vs. late-stage CRCs) (Supplementary Fig. S4a, c). Additionally, clusterin and cofilin-1 exhibited the highest FC of 1.5 in the study. In terms of overall differential protein levels, clusterin was decreased, while GSH-S and cofilin-1 were increased in platelets of CRC patients.

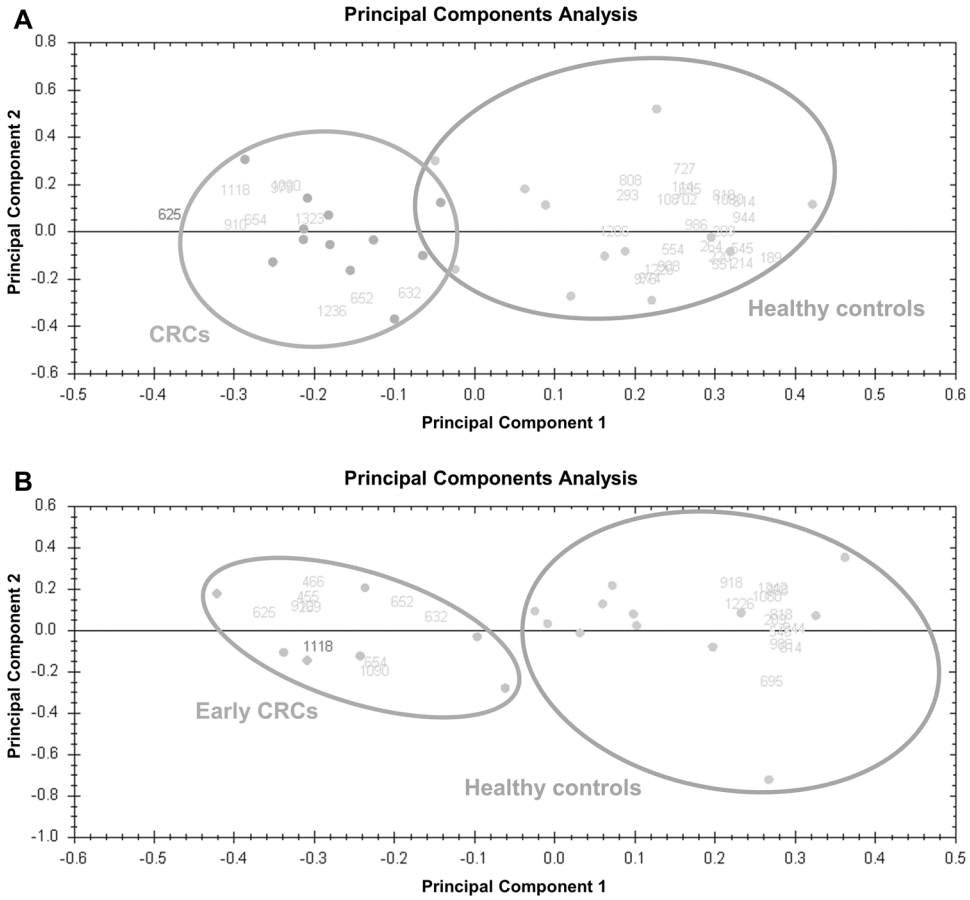

Fig. 2 Distinct group clustering of two-group comparisons by supervised PCA plotting. Supervised PCA plots between **a** healthy controls ($n = 12$) vs. CRCs (early-stage and late-stage, $n = 12$) based on 35 significant protein spots (t test, $p < 0.05$) and of **b** healthy controls ($n = 12$) vs. early-stage CRCs ($n = 7$) based on 22 differentially expressed protein spots (t test, $p < 0.05$). *Dots* represent platelet samples from each group

Validation of target proteins by Western blot

Validation of clusterin, GSH-S and cofilin-1 was performed by multiplex fluorescence-based Western blot analyses on a new cohort of 15 healthy volunteers, 15 early-stage CRC (UICC I and II) and 15 late-stage CRC (UICC III and IV) patients. Western blot data were in accordance with 2D-DIGE results and reached significances of $p < 0.05$ featuring either lower (clusterin) or higher levels (GSH-S, cofilin-1) in total CRCs, early-stage CRCs or late-stage CRCs compared to healthy controls (Fig. 4a–c). Moreover, cofilin-1 protein level was observed to be significantly different between early-stage CRCs vs. late-stage CRCs ($p = 0.0008$) as well as in the three-group comparison healthy controls vs. early-stage CRCs vs. late-stage CRCs ($p = 0.0062$). Concerning the comparison of healthy controls vs. early-stage CRCs, all three proteins exhibited significances of $p \leq 0.034$ in Western blot analyses. Grouping samples into healthy controls or early-stage CRC patients, highest sensitivity of 86.7% (at 40.0% specificity) and highest specificity of 93.3% (at 40.0% sensitivity) was achieved for clusterin, whereas GSH-S expression reached as best a sensitivity of 93.3% (at 46.7% specificity) and a specificity of 86.7% (at 46.7% sensitivity) (Fig. 4a, b; Supplementary Tables S5, S6). For cofilin-1, the highest sensitivity was obtained at 100% (with 53.3% specificity) and the highest specificity at 80.0% (with 46.7% sensitivity) between healthy controls and early-stage CRCs (Fig. 4c; Supplementary Table S7). Since all three proteins were statistically dependent in a multiple logistic regression model, a combination of the markers is not able to predict better group separation.

Discussion

In this study, the presence of discriminatory proteomic biomarkers in platelets of patients with colorectal carcinoma compared to healthy donors was demonstrated for the first time. Comprehensive platelet protein profiling by two-dimensional multiplex fluorescence gel electrophoresis detected 94 distinct spots which were significantly different between healthy controls, early-stage and late-stage CRC patients (Fig. 1). PCA-based cluster analyses revealed distinct separations between the groups (Fig. 2a, b; Supplementary Fig. S3a–c). A total of 54 platelet proteins were identified (Supplementary Table S3) and corresponding functional networks and canonical pathways were associated with, e.g., *Cellular function and maintenance*, *Cellular assembly*

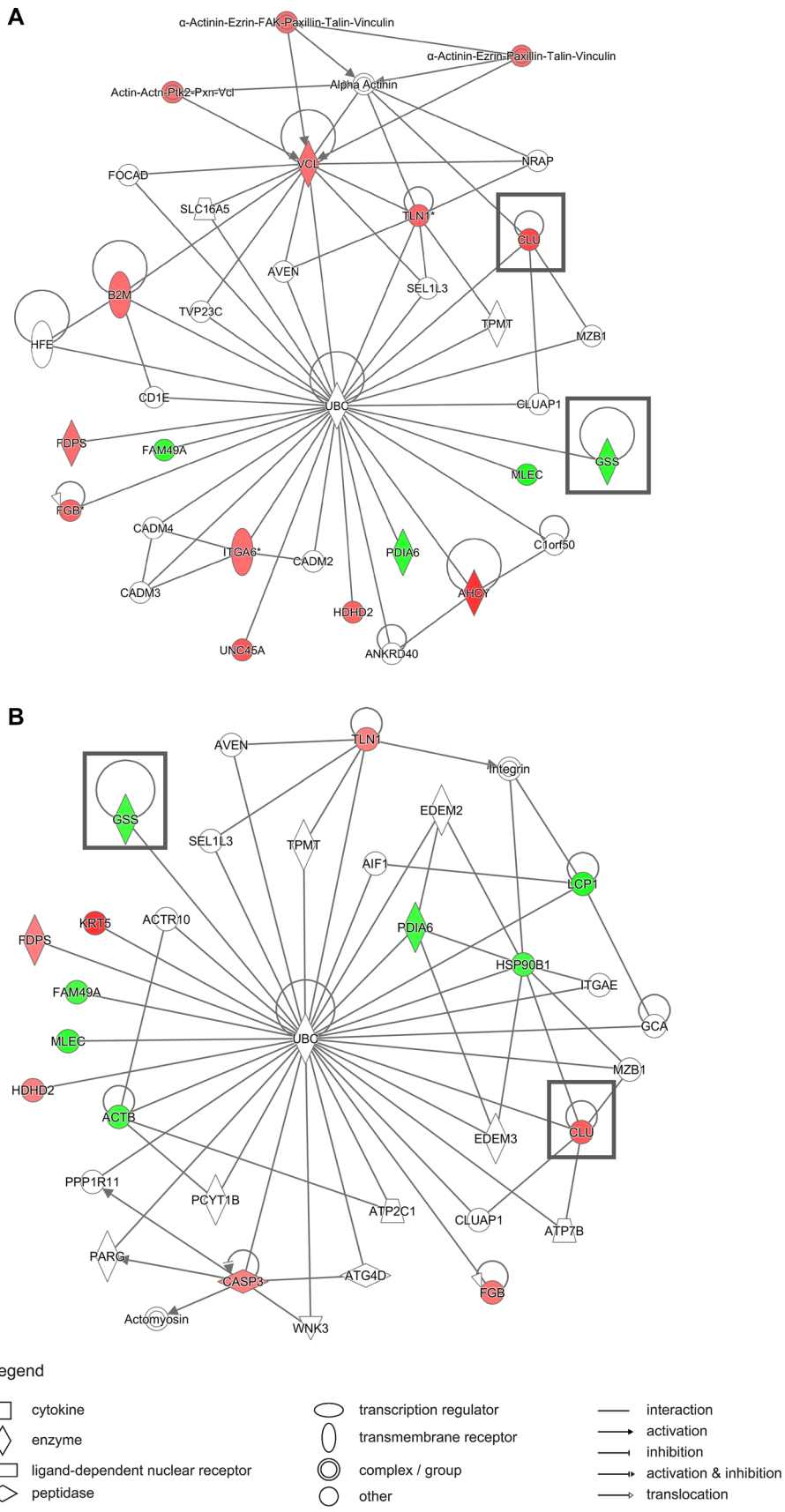

Fig. 3 IPA networks of direct relationships. IPA networks of 14 proteins differentially expressed **a** between healthy controls vs. CRC patients (early- and late-stage) with a score of 40 and **b** between healthy controls vs. early-stage CRCs with a score of 41. *Red* marked proteins are lower expressed in CRC platelet samples compared to healthy controls, whereas *green* highlighted proteins are higher expressed in cancer samples. Out of these networks, clusterin (gene symbol: CLU) and glutathione synthetase (gene symbol: GSS) were selected for subsequent validation

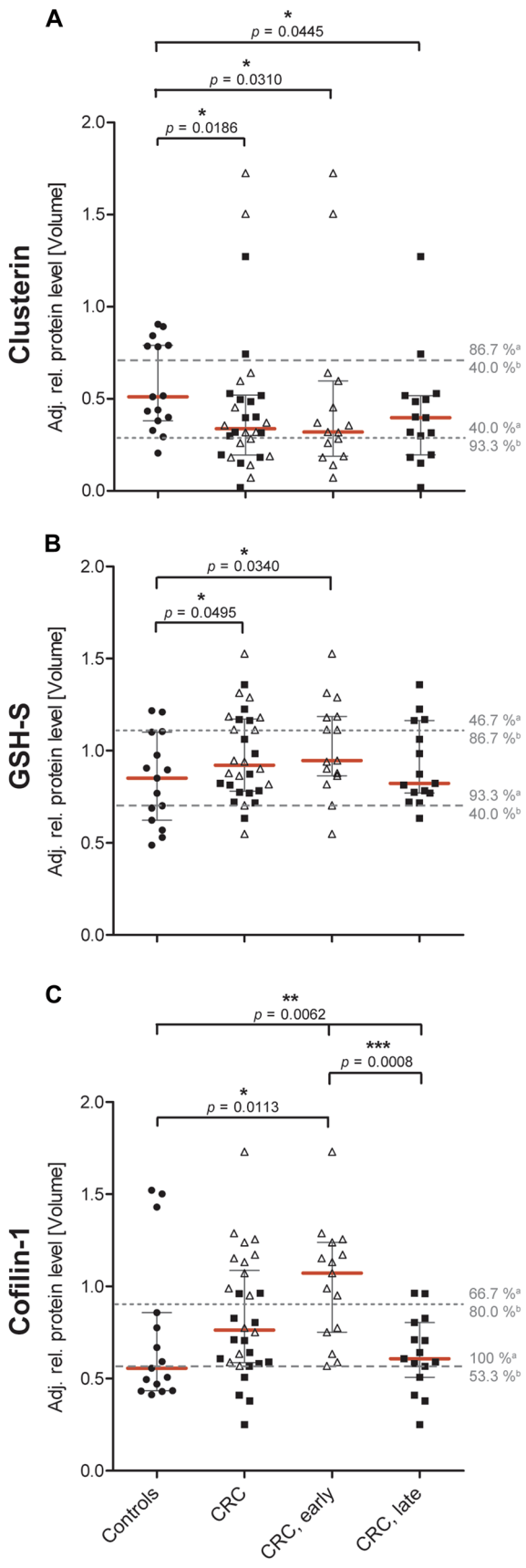

Fig. 4 Western blot validation of platelet target proteins clusterin, GSH-S, and cofilin-1. Specific antibody-targeted protein bands were detected by Cy3-labeled secondary antibody. Cy5 total protein signals within each lane were used for normalization (Cy3/Cy5 ratio). Based on an internal standard, adjusted relative protein-level calculation was performed. Western blot validation of selected target proteins **a** clusterin, **c** GSH-S, and **e** cofilin-1 confirmed significant different level characteristics obtained by 2D-DIGE between distinct groups ($p < 0.05$). Based on ROC curve calculation, cut-offs for clusterin, GSH-S, and cofilin-1 were selected grouping values of protein levels into healthy control or early-stage CRC patient. *Red lines* represent median values. Maximal sensitivity is indicated by *dashed lines* and best specificity by dotted lines. *a* sensitivity; *b* specificity

and organization, *Developmental disorder* and *Organismal injury and abnormalities* (Fig. 3a, b; Supplementary Table S4). Subsequent validation by multiplex fluorescence-based Western blot analyses using an independent cohort of platelet protein samples confirmed significant different 2D-DIGE level characteristics of three target proteins—clusterin, GSH-S and cofilin-1. While clusterin was significantly decreased, GSH-S and cofilin-1 showed significant higher protein levels in platelets from CRC patients compared to healthy donors (Fig. 4a–c). Grouping healthy controls and early-stage CRC patients, maximal sensitivity values ranged from 86.7% (clusterin) to 100% (cofilin-1) at 40.0–53.3% specificity. Best specificity values were determined between 80.0% (cofilin-1) and 93.3% (clusterin) at 40.0–66.7% sensitivity (Fig. 4a–c).

In vitro activation of platelet samples and release of secretory granules during blood handling and isolation was excluded (Supplementary Fig. S2) to ensure actual in vivo physiological or pathological states for all proteomic experiments.

Clusterin is a heterodimeric glycoprotein, ubiquitously expressed in epithelial cells of mammalian tissues and secreted to physiological fluids. As a stress-induced and cell-protecting extracellular chaperone, clusterin is involved in apoptotic cell death processes during developmental and pathological states [35, 36]. Upregulation of clusterin protein levels were detected during tumor progression and clusterin expression directly correlates with tumor aggressiveness and metastatic potential of the tumor. Clusterin is extracellularly released by colorectal cancer cells into the blood and is also present in platelet α-granules from where it is released upon activation during direct interaction between tumor cells and platelets [35, 37, 38]. Therefore, decreased clusterin levels in platelets of CRC patients even at early malignant stages—as investigated in this study—could be caused by an activated status and modulation of the platelet protein content for contribution of cancer progression.

GSH-S is expressed in blood and nucleated cells and occurs as a homodimer. It is a metabolizing enzyme involved in the glutathione biosynthesis pathway catalyzing the ATP-dependent formation of glutathione (GSH) from

γ-glutamylcysteine and glycine. GSH provides protection from cancer while playing a crucial role during protection against oxidative stress, which in turn promotes cancer development and progression [39]. GSH-S is expressed at higher levels in colon cancer tissue as compared with normal mucosa and therefore reported to be a potential clinical useful biomarker of colon cancer and target for anti-colon cancer drugs [40]. In addition, it was hypothesized that the potential value of examining the regulation of glutathione synthesis may serve as an indicator of clinical prognosis for malignancy [41]. Our observed increased GSH-S enzyme levels in CRC platelets are in line with the literature and strengthen the impact of GSH-S as pro-tumorigenic factor favoring primary tumor proliferation.

The non-muscle isoform cofilin-1 is a cytoskeletal protein which is ubiquitously expressed in eukaryotic cells [42]. The fundamental function of cofilin-1 is accelerating the turnover of actin filaments by depolymerizing or serving actin filaments and is essential for regulation of actin dynamics, cell division, cell migration and chemotaxis. It is also speculated that cofilin-1 and DNA interaction may influence various biological responses, including DNA damage repair [43]. Cofilin-1 overexpression is reported to be directly associated with the invasion, metastasis and chemoresistance in several types of malignancies, e.g., prostate, breast, ovarian, bladder, pancreatic and non-small cell lung cancer [42, 44–50]. Since circulating platelets are involved in cytoskeletal-dependent processes such as tumor progression, angiogenesis, cancer cell protection, extravasation and metastasis [6–9], elevated cofilin-1 protein levels in our study may have impact on these cancerous processes. We detected the platelet protein level of cofilin-1 to be higher in early-stage CRC patients than in healthy controls with 100% sensitivity.

We assume a close association between early-stage CRCs, platelets and three target proteins. Significant different protein levels of clusterin, GSH-S and cofilin-1 between platelets from healthy controls and CRC patients reflect the actual and highly dynamic pathophysiological state of the CRC disease and may serve as additional diagnostic biomarkers. This might be particularly true and of high clinical relevance for the early detection of CRC. Liquid biopsy can be of high impact for biomarkers in early CRC diagnosis, therapy guidance, surveillance and disease monitoring. Our platelet protein markers individually detect early-stage CRCs between 86.7 and 100% sensitivity with 40.0–53.3% specificity and are thus comparable to current screening methods in clinical application (e.g., immunochemical fecal occult blood testing [iFOBT], M2-pyruvate kinase [M2-PK] fecal test) [51–53]. However, even these CRC screening tests are insufficient in detecting pre-malignant stages such as polyps and adenomas. In this case, invasive colonoscopy still remains gold standard. Since neither acceptance of colonoscopy in population nor compliance and diagnostic performances of iFOBT or M2-PK fecal tests alone seem satisfying for early detection of CRC today, we believe that a combination of further appropriate minimal-invasive tumor markers with established methods may improve the efficiency of early-stage CRC screening in the future. Here, platelet proteome studies promise to provide potential target proteins associated with malignant genesis and progression. In this context, platelet protein markers of pre-malignant CRC developmental stages afford the opportunity to improve the CRC screening and should be the subject of further research. Prospective multicenter trials respecting the intra- and inter-subject diversity of the proof-of-concept study are warranted.

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Compliance with ethical standards

Ethical standards This study was approved by the local Ethics Committee of the University of Lübeck (#07-124). Blood samples of CRC patients and healthy volunteers were processed after informed consent and stored at the Interdisciplinary Centrum for Biobanking-Lübeck (ICB-L). The experiments comply with the current laws of the country in which they were performed.

Conflict of interest The authors declare that they have no conflicts of interest.

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