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ORIGINAL ARTICLE

Prognostic value of DLGAP5 in colorectal cancer

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Abstract

Purpose DLG7 (disc large homolog 7) is a microtubule-associated protein encoded by *DLGAP5* (DLG associated protein 5) gene and has an important role during spindle assembly. Spindle assembly deregulation is a well-known cause of genomic instability. The aim of this study was to investigate the influence of *DLGAP5* expression on survival and to evaluate its potential use as a biomarker in colorectal cancer (CRC).

Methods *DLGAP5* expression was measured in the primary tumor and corresponding normal mucosa samples from 109 patients with CRC and correlated to clinical and pathological data. The results were validated in a second, publically available patient cohort. Molecular effects of DLG7/*DLGAP5* in CRC were analyzed via functional assays in knockdown cell lines.

Results *DLGAP5* downregulation led to a significant reduction of the invasion and migration potential in CRC. In addition, *DLGAP5* expression correlates with nodal status and advanced UICC stage (III–IV).Subgroup analyses revealed a correlation between *DLGAP5* overexpression and poor survival in patients with non-metastatic disease (M0). Furthermore, overexpression of *DLGAP5* is associated with worse overall survival in distinct molecular CRC subtypes.

Conclusions The results of this study suggest the importance of *DLGAP5* in defining a more aggressive CRC phenotype. DLG7/*DLGAP5* represents a potential biomarker for CRC in molecular subgroups of CRC.

Keywords DLG7 · *DLGAP5* · Colorectal cancer · Metastasis · Survival

Introduction

Colorectal cancer (CRC) is one of the three most frequent malignant diseases and represents a molecularly heterogeneous group characterized by a large number of genomic and epigenomic alterations [1, 2]. Despite advances in understanding CRC tumorigenesis, there is still an urgent need for potential molecular biomarkers and therapeutic targets.

DLG7 (disc large homolog 7) is a microtubule-associated protein (MAP) encoded by the *DLGAP5* (DLG associated

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protein 5) gene and has a crucial role during spindle assembly, kinetochore fibers (K-fibers) stabilization, and chromosomal segregation during mitosis [3–6]. DLG7 is stabilized via phosphorylation by Aurora A kinase and plays a key role in centrosome formation and chromosome segregation, thus regulating the formation of the spindle apparatus [7, 8].

DLGAP5 overexpression has been found in CRC, breast cancer, hepatocellular carcinoma, urinary bladder cancer, meningioma, and adrenocortical cancer [3, 9–13]. Previous studies have already described an association between *DLGAP5* deregulation and abnormal chromosomal rearrangement [5, 14]. Rearrangement errors due to spindle assembly deregulation are well-known causes of genomic instability [15, 16]. Thus, *DLGAP5* overexpression may contribute to carcinogenesis.

Our study is the first to describe the expression of *DLGAP5* in a large cohort of CRC patients and its relation to survival. In order to investigate the influence of *DLGAP5* expression in CRC biology, we performed functional assays in knockdown CRC cell lines and demonstrated the influence of *DLGAP5* overexpression on cancer aggressiveness in vitro. To unveil the potential use of CRC as a biomarker, we performed a subgroup survival analysis in an external CRC cohort and found a correlation between *DLGAP5* expression and survival in non-metastatic CRC.

Material and methods

Patients and tissue samples

Primary tumor and corresponding normal tissue (i.e., colonic mucosa) samples were collected from 109 patients with CRC who underwent tumor resection at the Department of General, Visceral and Transplantation Surgery at the University Hospital in Heidelberg between 2009 and 2012. Informed consent was obtained prior to surgery. The study was approved by the independent ethics committee of the University of Heidelberg (Ethics committee number 323/2004).

Affymetrix CRC cohort

A database of publicly available colorectal cancer patient samples with corresponding microarray expression data by Affymetrix gene chips was set up as previously described [17]. The Affymetrix CRC cohort consists of 550 CRC patients of all stages. Detailed clinical characteristics of all patients including gender, stage, grade, MSI, and location are displayed in Supplemental Table S1. The probe 203764_at was utilized for DLGAP5. Survival analysis was performed by Cox proportional hazard regression in the R statistical environment (www.r-project.org) using the best cutoff as previously described [18, 19]. To visualize differences, Kaplan–Meier survival plots, HR (hazard ratio) with 95% CI (confidence intervals), and logrank *p* value were plotted using the library "survival." We also analyzed the Affymetrix cohort according to UICC stages and different molecular subtypes. The following molecular subtypes were included in the analysis:

Colon cancer hypoxia score

Dekervel et al. [20] identified a prognostic score (Colon Cancer Hypoxia Score (CCHS)) in patients with stages II and III colon cancer. Patients with a high expression of hypoxia-related genes (CCHS-high) have a worse overall survival (OS), whereas patients in the CCHS-low group (low expression of hypoxia-related genes) have a better prognosis.

Meta 163 Dukes stages B and C cancer

Jorissen et al. [21] identified a panel of 128 genes capable to classify Dukes stages B and C cancer in patients with a good prognosis (stage A like) and patients with poor prognosis (Stage D like). Although the Dukes classification is no longer used in the clinical practice, it is still of historical value.

Budinska subgroups

Budinska et al. [22] identified five CRC subtypes (surface crypt-like, lower crypt like, CIMP-H like, mesenchymal, and mixed type) according to genome-wide expression patterns.

Oncotype DX assay

The Oncotype DX assay is a RT-PCR (real-time polymerase chain reaction)-based test that can estimate the risk of recurrence in stage II or III colon cancer [23].

OncoDefender subgroups

OncoDefender is a RT-PCR-based test optimized for FFPE (formalin-fixed paraffin-embedded) samples to predict recurrence in UICC stage I rectal cancer and in UICC stages I and II colon cancer [24]

Merlos-Suarez subgroups

Merlos-Suarez et al. [25] identified an intestinal stem cells (ISCs) gene signature which predicts cancer relapse. ISCs cells are characterized by a high expression of the EphB2 receptor, which become progressively silenced along with cell differentiation. The authors used ISC gene expression levels to stratify CRC patients into three groups (high, medium, and low ISC signature). CRC patients with high expression of ISC genes had a 10-fold higher relative risk of relapse compared with CRC patients with low ISC genes levels ($p < 0.0001$). The EphB2-ISC medium expression group displayed an intermediate risk.

CIN25

Carter and colleagues [26] developed a chromosome instability gene signature (CIN25), based on 25 genes that are most overexpressed in CIN tumors. Patients with a negative CIN signature have a better prognosis.

Cell culture

Colorectal cancer cell lines DLD1, HT29, HCT116, SW480, SW620, and Colo205 were obtained from ATCC. As a control, the commercial cell line NHDF (Normal Human Dermal Fibroblast; PromoCell, Germany) was used. The cells were incubated at 37 °C with 5% CO_2 and 95% humidity. DMEM and RPMI (PAA, Germany) or fibroblast growth media (PromoCell, Germany) were supplemented with 10% fetal bovine serum (FBS; PromoCell, Germany), 1% penicillin/streptomycin, and 1% L-glutamine (Life Technologies, USA). Cell lines were regularly screened for mycoplasma contamination with a PCR-based test. Cells were maintained in T75 flasks (Corning Glass, USA). Cultures were split (1:5) when they reached 75–90% confluence using Accutase (PAA, USA).

RNA isolation

The RNeasy Kit (Qiagen) was used for RNA isolation following the manufacturer's instructions. Briefly, tissue fragments were inserted into microcentrifuge tubes containing 700-μL buffer RA1 and one 5-mm stainless steel bead (Qiagen, Germany). Samples were shaken twice at 30 Hz for 2 min (TissueLyser II, Qiagen, Germany). Cell line pellets were lysed with 350-μL buffer RA1. RNA was eluted with 40 μL RNase-free water, and the concentration was measured using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, USA). The same kit was used for the RNA isolation from cultured cells.

cDNA synthesis

The High-Capacity cDNA Reverse Transcription Kit (Promega, USA) was used for cDNA synthesis. The probes were incubated in a thermocycler (Eppendorf, Germany) at 25 °C for 10 min, at 45 °C for 60 min, and at 70 °C for 15 min. Tissue samples were diluted with nuclease-free water (Qiagen, Germany) in a 1:5 ratio and cell lines in a 1:10 ratio.

Quantitative PCR

Quantitative PCR was performed using the LightCycler®480 Real-Time PCR System (Roche, Switzerland). One reaction consisted of 2.5 μL cDNA, 5 μL SYBR® Green PCR Master Mix, 0.5 μL from each 5 μM primer (forward and reverse), and 1.5 μL RNAase-free water (Qiagen, Germany). The following primers were used (5′ to 3′): *DLGPA5*_fwd CGACCTGGTCCAAGACAAAC, *DLGAP5*_rev GCTGCTTGAGTAGCTGATCG, *GAPDH*_fwd GACCCCTTCATTGACCTCAAC, *GAPDH*_rev TTGATTTTGGAGGGATCTCG, *SDHA*_fwd TGATCTTCGCTGGCGTGGAC, *SDHA*_ rev CCGGGCACAATCTGATCCTG, *CYC1*_fwd TGTTCATGCGGCCAGGGAAG, *CYC1*_rev TGAGCAGGGAGAAGACGTAG.

Holding stage was set at 50 °C for 2 min and at 95 °C for 5 min. Cycling stage was set at 95 °C, 60 °C, and 72 °C, and the reaction was repeated 45 times. Melting curve stage was set at 95 °C for 5 min and ramped up with a 0.2 °C increase from 65 °C to 97 °C. All samples were measured in triplicate, and the mean of the Ct values was calculated. All values were normalized to three widely used and robust housekeeping genes (*GAPDH*, *CYC1*, *SDHA*) with the formula: $\Delta Ct = Ct_{(DLGAP5)} - Ct_{(\mathrm{housekeeping\ genes})}$. The ΔΔCt method was used to determine the relative expression of *DLGAP5* in the tumor samples. DLGAP5 expression in tumor tissue was normalized to the corresponding mucosa, and results were displayed as ΔΔCt values: $\Delta\Delta Ct = \Delta Ct_{\mathrm{tumor}} - \Delta Ct_{\mathrm{normal\ tissue}}$, where $\Delta Ct_{\mathrm{tumor}} = Ct_{DLGAP5\ \mathrm{tumor}} - Ct_{\mathrm{reference\ genes\ tumor}}$ and $\Delta Ct_{\mathrm{normal\ tissue}} = Ct_{DLGAP5\ \mathrm{normal\ tissue}} - Ct_{\mathrm{reference\ genes\ normal\ tissue}}$. Ct values from all cell lines were normalized to the reference genes. The gene fold change was calculated using the $2^{\Delta Ct}$ equation. *DLGAP5* transcript level fold in cell lines was calculated using the formula $2^{\wedge}[Ct_{(DLGAP5)} - Ct_{(\mathrm{housekeeping\ genes})}]$.

Protein extraction and western blot

Protein extraction was carried out using SDS Buffer (Sigma-Aldrich, USA) with the addition of protease inhibitor (Roche, Switzerland). For 1×10^6 cells, 400 μl of SDS buffer was used. The cell lysis products were sonicated and centrifuged at 4 °C for 15 min. Sample concentrations were determined using a BCA kit (Thermo Scientific, USA), the absorbance was measured with a multi-well spectrophotometer (Infinite Reader F200 Pro with Magellan Software, Tecan, Switzerland). Western blots were performed using 60 μg of protein per sample. Transfer was performed for 1 h at room temperature. The nitrocellulose membrane was then incubated at 4 °C overnight, with the primary antibodies at a dilution of 1:10000 (DLG7 Rabbit Antibody, Bethyl Laboratories, USA) and 1:1000 (β-tubulin rabbit antibody, Cell Signaling Technology, USA). The secondary antibody (HRP IgG rabbit antibody, Cell Signaling Technology, USA) was then incubated at 4 °C for 2 h at a 1:1000 dilution. A chemoluminescence detection was performed using Pierce ECL Western Blotting-Substrate (Thermo Fisher Scientific, USA).

Cell proliferation assay

A total of 1×10^3 cells were seeded in each well of a 96-well plate and incubated at 37 °C in 100 μl of appropriate medium for 24 h. After 24 h, 48 h, 96 h, and 120 h of incubation, a 100-μl solution of 90-μl medium and 10-μl cell proliferation reagent WST-1 (ScienCell, USA) were added. After 30 min of incubation at 37 °C, the absorbance was measured using a multi-well spectrophotometer (Infinite Reader F200 PRO with Magellan Software, Tecan). Each experiment was repeated three times.

DLGAP5 knockdown

Three colon cancer cell lines (DLD1, HCT116, and SW480) were arbitrarily chosen for transfection. Cells were transfected with pGFP-V-RS vectors (OriGene Technologies Inc., USA) using Lipofectamine 2000 (Invitrogen/Life technologies, USA) allowing stable delivery of the shRNA expression cassette against *DLGAP5.* After 24 h, transfected cells were selected with puromycin (final concentration, 10 μg/ml). Control cell lines were created using a control pGF-V-RS which is a vector lacking the shRNA expression cassette for *DLGAP5*. A limiting dilution cloning protocol was used to select cell clones.

Plasmid amplification

One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen/Life Technologies, USA) were transformed following the manufacturer's instructions using 2 μL from the corresponding plasmids. Plasmid DNA was isolated with the Qiagen Maxi Kit following the manufacturer's protocol. DNA was eluted with nuclease-free water at a final concentration of 1 μg/μL.

Invasion and migration assays

Matrigel invasion chambers (BD Biosciences, USA) were used for the invasion assay. Matrigel invasion chambers consist of an insert containing an 8-μm pore size membrane coated with Matrigel. The matrigel layer occludes the pores and simulates a basement membrane. Only invasive cells are able to pass through the Matrigel layer.

Migration chambers (Greiner Bio-One, Germany) consist of a membrane with 8-μm pores and used to assess cell migration response to a chemoattractant.

In every chamber, 50,000 knockdown or control cells were seeded in serum-free medium. For each cell line, three chambers were used. Each chamber was put in a suitable well filled with medium and 10% FCS as a chemoattractant for the invasion assay (HCT116: medium with 20% FCS). DMEM medium with the addition of 20% FCS (5% for DLD1) was used for the migration assays as a chemoattractant.

After 24 h of incubation, the medium was removed; the cells were fixed with methanol, stained with a 2.3% crystal violet solution (Sigma-Aldrich, USA), and counted under a microscope (DM IL LED, Leica, Germany). The experiment was repeated three times for each cell line.

Statistical analysis

Statistical analysis and graphics were performed with SPSS Statistic 22 (IBM, USA) and Prism 6 (GraphPad Software, USA). Kaplan–Meier curves were used to illustrate survival rates. The logrank test was used to compare survival curves. Subgroup survival analyses were made for UICC (Union for International Cancer Control) stages and nodal status (positive and negative nodal status, Fisher's exact test). Results from in vitro experiments were analyzed using the Student's t test and Fisher's exact test. $p < 0.05$ was considered statistically significant.

Results

DLGAP5 is overexpressed in colorectal cell lines

A significant *DLGAP5* overexpression was found in CRC cell lines DLD1 (1.1 ± 0.9 vs. 0.4 ± 0.1; $p < 0.001$), SW480 (1.4 ± 0.2 vs. 0.4 ± 0.1; $p < 0.001$) and HCT116 (0.8 ± 0.1 vs. 0.4 ± 0.1; $p < 0.05$) in comparison with the non-malignant control cell line NHDF. An expression of DLG7 at the protein level was also found in almost every cell line. Results for gene expression analysis and western blot are displayed in Fig. 1 a and b, respectively.

Changes in DLGAP expression modulates proliferation, migration, and invasion behaviors of colorectal cancer cell lines

To study the effects of DLG7/*DLGAP5* on tumor biology, stable knockdown transfections in three representative CRC cell lines (DLD1, HCT116, and SW480) were obtained. *DLGAP5* knockdown leads to a statistically significant reduction in the proliferation of DLD1 (OD mean ± SEM, 0.46 ± 0.03 vs. 0.24 ± 0.01; $p < 0.001$) and HCT116 (OD mean ± SEM, 0.5 ± 0.03 vs. 0.33 ± 0.04; $p < 0.01$, Fig. 1c). Surprisingly, knockdown of *DLGAP5* resulted in an increase in proliferation in SW480 cells (OD mean ± SEM, 1.11 ± 0.03 vs. 1.46 ± 0.02; $p < 0.0001$). The cell cycle analysis showed no difference between knockdown cell lines and controls (Supplemental Fig. S1). In the CRC cell lines, a diminished migratory (Fig. 1d) and invasive potential (Fig. 1e) was observed after *DLGAP5* knockdown. The most striking decrease in migration (93.1%) was seen in DLD1 cells (1254 ± 136 cells vs. 86 ± 32 cells; $p < 0.05$); in HCT116, migration decreased by 47.9% (315 ± 24 vs. 164 ± 4; $p < 0.01$) and in SW480 by 81.8% (154 ± 9 vs. 28 ± 3; $p < 0.001$). A 70.2% decrease in invasion ability was observed in DLD1 cells (990 ± 203 vs. 295 ± 58; $p < 0.05$) and a 71.3% decrease in invasion ability in SW480 cells (94 ± 15 vs. 27 ± 8; $p < 0.05$) after

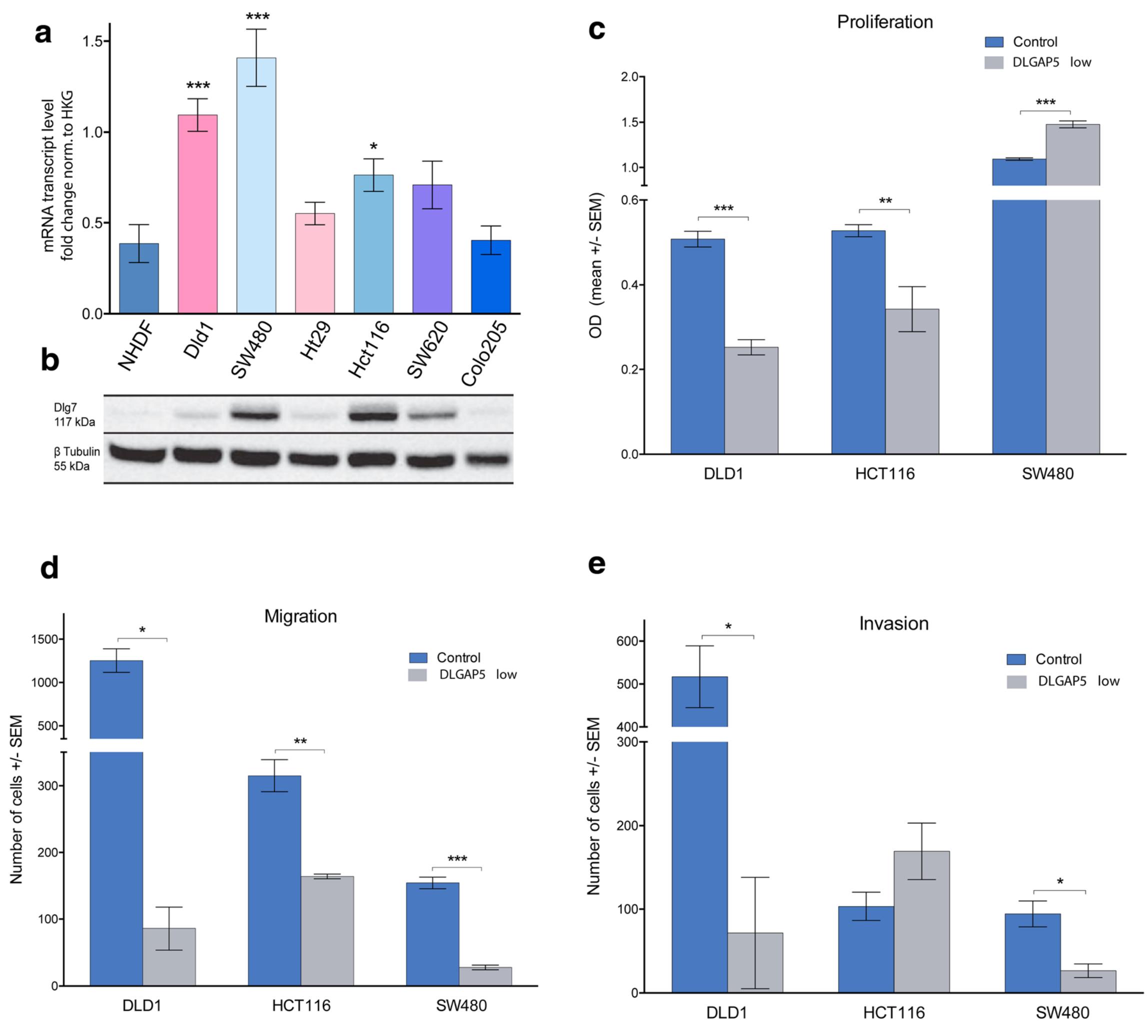

Fig. 1 Normalized *DLGAP5* expression (fold change) in CRC cell lines compared with the non-malignant cell line NHDF (**a**). Western blot for DLG7 protein (117 kDa) and β-Tubulin (55 kDa) (**b**). Proliferation (**c**), migration (**d**), and invasion assays (**e**) of CRC cell lines after *DLGAP5* downregulation. Means and ± SEM (standard error of the mean) are displayed. **p* < 0.05, ***p* < 0.01, ****p* < 0.001

DLGAP5 knockdown. No differences on invasion behavior were detected in HCT116 upon *DLGAP5* knockdown. The results discussed in these two sections are summarized in Fig. 1.

DLGAP5 is a negative prognostic factor in CRC

DLGAP5 transcripts were measured in the primary tumor and normal tissue samples from 109 CRC patients. *DLGAP5* expression in tumor tissue was normalized to the corresponding mucosa and results were displayed as ΔΔCt values (Fig. 2a). Clinical and pathological data are summarized in Table 1. Patients were separated into two cohorts according to a high or low expression of *DLGAP5*. A ΔΔCt value > 1 was chosen as cutoff for high *DLGAP5* expression. In most of the tissue samples from CRC patients, overexpression of *DLGAP5* was observed, resulting in a cohort of 82 patients with high *DLGAP5* expression and a cohort including 27 patients with low *DLGAP5* expression. In our cohort, *DLGAP5* overexpression led to worse overall survival (OS) as shown in the Kaplan–Meyers curves (Fig. 2b), especially in patients with UICC stage IV CRC and in patients with negative nodal status. However, this difference was not statistically significant.

DLGAP5 expression correlates with nodal status in patients with CRC

A correlation between lymph node status and *DLGAP5* expression was found. In 48.8% (40/82) of patients with intratumoral *DLGAP5* overexpression, a positive nodal status was observed, as opposed to 25.9% (7/27) of the patients with low *DLGAP5* expression. This correlation between nodal status (N status) and *DLGAP5* expression was statistically significant (OR 2.72; 95% CI

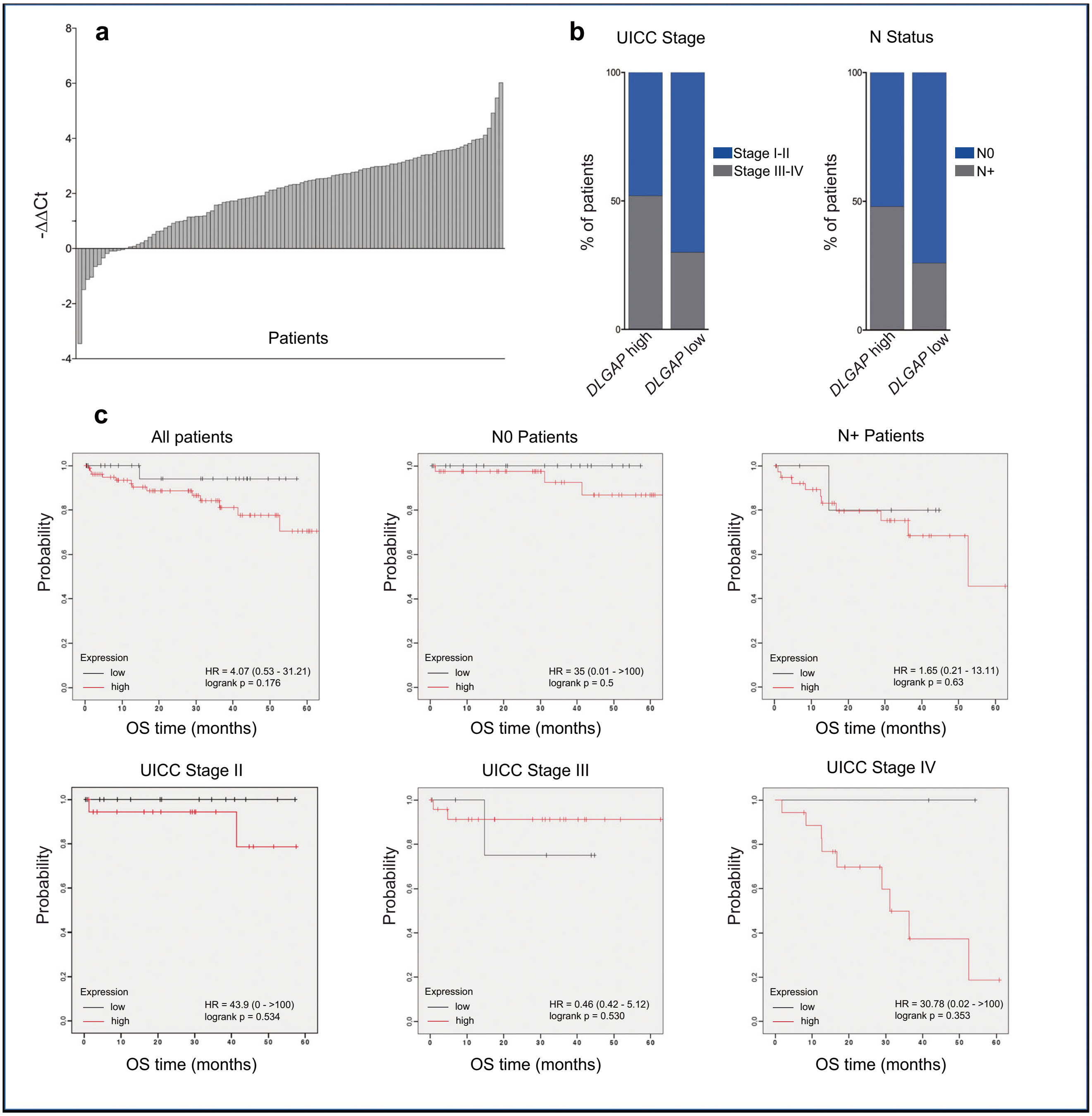

Fig. 2 Relative *DLGAP5* expression in tissue from 109 CRC patients. Every column represents a patient. **a** Comparison of overall survival (OS) in patients with (green line) or without (blue line) *DLGAP5* overexpression, according to N status, UICC stage (Union for International Cancer Control) (**b**). C_t, threshold cycle; N0, negative nodal status; N+, positive nodal status. In the UICC stage I group, no event of interest (death) was recorded; therefore, a statistical analysis within this subgroup was not possible and Kaplan–Meyer curves are not displayed. Patients with advanced disease or positive N status (percentage). Patients are divided into *DLGAP5* high or *DLGAP5* low, depending on *DLGAP5* expression (**c**)

1.04–7.13; $p < 0.05$). A significant association between advanced UICC stage (III–IV) and *DLGAP5* overexpression (OR 2.62; 95% CI 1.03–6.66; $p < 0.05$) was also found. In 52.4% (43/82) of patients with an advanced UICC stage, a *DLGAP5* overexpression was observed (Fig. 2c).

DLGAP5 expression is a negative prognostic factor predominantly in non-metastatic disease with a high risk of recurrence

Upon subgroup analysis, an effect of *DLGAP5* on survival was found in patients with non-metastatic disease (M0).

Table 1 Patient characteristics

		Total		*DLGAP5* low		*DLGAP5* high	
		N	%	*N*	%	*N*	%
Patients		109	100	27	24.8	82	75.2
Age	Median	65		63		65	
	Range	35–88		35–79		35–88	
Gender	F	48	44	12	25	36	75
	M	61	56	15	24.6	46	75.4
Localization	Colon	46	42.2	12	26.1	34	73.9
	Rectum	62	56.9	15	24.2	47	75.8
	Unknown	1	0.9	0	0	1	100
N	N0	62	56.9	20	32.2	42	67.7
	N+	47	43.1	7	14.9	40	85.1
UICC	I	23	21.1	4	17.4	19	82.6
	II	35	32.1	15	42.9	20	57.1
	III	31	28.4	6	19.3	25	80.7
	IV	20	18.3	2	10	18	90
Grade	Unknown	12	11	6	50	6	50
	2	81	74.3	19	23.5	62	76.5
	3	16	14.7	2	12.5	14	87.5

High *DLGAP5* expression (upper quartile of expression was used as a cutoff) was associated with poor survival (HR 2.45; interquartile range (IQR) 1.17–5.12; logrank $p = 0.014$) (Fig. 3a, b).

In the low-risk CCHS group, DLGAP5 overexpression is associated with poor OS (HR 2.23; IQR 1.1–4.54; logrank $p = 0.023$) (Fig. 3c).

DLGAP5 overexpression is associated with poor prognosis in stage A–like group (HR 2.58; IQR 1.12–5.98; logrank $p = 0.021$) (Fig. 3d).

The Budinska subtypes A (surface crypt like) (Fig. 3e) and B (low crypt like) (Fig. 3f) were found to have the best prognosis compared with the other subgroups. In the subtype B, *DLGAP5* overexpression is strongly associated with poor prognosis (HR 2.81; IQR 1.42–5.55; logrank $p = 0.002$), whereas in subtype A, *DLGAP5* overexpression is associated with a better prognosis (HR = 0.38; IQR 0.15–0.92; logrank $p = 0.027$). In the Oncotype DX subgroup with high recurrence risk, a *DLAGAP5* overexpression is related to poor prognosis (HR 1.73; IQR 1–3; logrank $p = 0.049$) (Fig. 3g). Contrarily, in the OncoDefender high-risk group, DLGAP5 correlated with better prognosis (HR 0.59; IQR 0.38–0.92; logrank $p = 0.02$) (Fig. 3h).

DLGAP5-related survival strongly depends on intestinal stem cell signature in CRC

In our analysis of the Affymetrix cohort, we noticed that a *DLGAP5* overexpression correlates with poor prognosis in the subgroup of CRC patients with no ISCs signature (HR = 3.09; IQR 1.38–6.91; logrank $p = 0.0038$) (Fig. 3i). On the other hand, in the subgroup with high ISCs signature, *DLGAP5* overexpression is associated with a better prognosis (HR = 0.4; IQR 0.18–0.92; logrank $p = 0.026$) (Fig. 3j).

Patients without chromosome instability and overexpression of DLGAP5 have a better survival

Patients with a negative CIN signature have a better prognosis. In this group of patients, we found that DLGAP5 overexpression is related to a better prognosis (HR = 0.56; IQR 0.34–0.92; logrank $p = 0.021$) (Fig. 3k).

Discussion

We here demonstrate that *DLGAP5* is overexpressed in colorectal cancer and, despite opposite effects in some subgroups, a negative prognostic factor for most CRC patients. Previous data demonstrated that *DLGAP5* is downregulated during the stem cell differentiation process; it is highly expressed in bone marrow precursor cells but not expressed in peripheral blood monocytes [27]. CRC progression is also believed to be a process originating from multipotent cancer stem cells [28]. We therefore hypothesized that *DLGAP5* may play a role in the pathogenesis of CRC.

The *DLGAP5* expression profile in CRC has not been systematically investigated yet. Bassal et al. reported *DLGAP5* overexpression in a few colon and breast cancer samples [9]. *DLGAP5* overexpression has also been demonstrated in hepatocellular carcinoma, urinary bladder transitional cell carcinoma, prostate cancer, meningioma, and adrenocortical carcinoma [3, 10–13, 29]. In the present study, we demonstrate that *DLGAP5* is overexpressed in both colorectal cell lines and tissue samples of CRC at mRNA and protein levels.

Tsou and colleagues demonstrated that *DLGAP5* overexpression increases the proliferation potential of human cells. We were also able to show that *DLGAP5* downregulation has a negative impact on the proliferation of CRC, which is well in line with previous studies in other tumor entities [3, 30]. DLG7/*DLGAP5* is involved in the stabilization of the oncoprotein Gankyrin, which has a role in the ubiquitination and degradation of p53 [31]. *DLGAP5* overexpression leads to Gankyrin accumulation and therefore to ubiquitin-mediated p53 degradation [30], which may explain the effects of DLG7/*DLGAP5* on proliferation. To further define the role of *DLGAP5* in the carcinogenesis of CRC, we investigated the effects of *DLGAP5* downregulation on invasion and migration. *DLGAP5* knockdown leads to a significant reduction of the invasion and migration potential in CRC in most cell lines. The discordant results observed in SW480 cells suggest a multifactorial influence between *DLGAP5* and cell

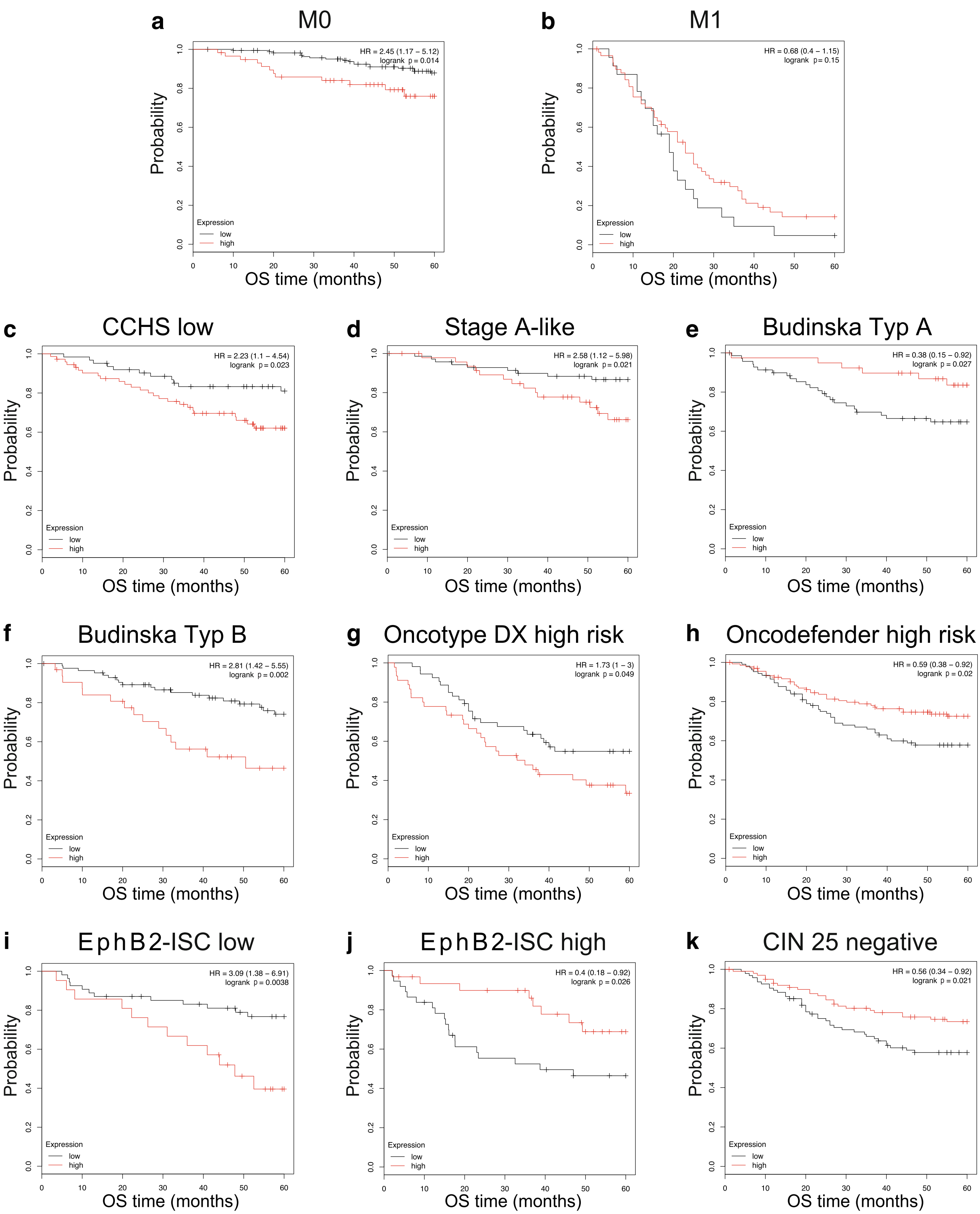

Fig. 3 Comparison of overall survival (OS) in patients with (red line) or without (black line) *DLGAP5* overexpression, according to nodal status, metastatic disease, and molecular subgroups (Affymetrix cohort). HR, hazard ratio; CCHS, Colon Cancer Hypoxia Score; EphB2-ISC, EphB2 receptor–intestinal stem cell signature; CIN, chromosome instability

migration and invasiveness. However, 48.8% (40/82) of the patients with a high *DLGAP5* expression had lymph node metastases. In addition, a significant association between *DLGAP5* overexpression and advanced UICC stage was observed. This clinical observation is well in line with the increased *DLGAP5*-related invasiveness we demonstrated in vitro.

Relation between high *DLGAP5* expression and impaired survival has been demonstrated in several tumor entities [10, 12, 32]. Despite a clear tendency towards impaired OS upon *DLGAP5* overexpression, this relationship failed to reach statistical significance in our patient cohort.

In order to externally validate our findings, we analyzed a large, publicly available cohort of CRC patients of all stages with associated microarray expression profiles. Again, we were unable to demonstrate a statistically significant association between DLGAP5 expression and OS. We thus hypothesized that *DLGAP5* overexpression has differential effects in different subgroups of CRC, which is also in line with our discordant findings in vitro, and performed numerous subgroup analyses.

DLGAP5 overexpression was a negative prognostic factor for OS in non-metastatic (M0) patients, but not M1 patients. As *DLGAP5* increases invasiveness and metastasis, its effects are larger in non-metastatic patients, who may ultimately develop distant metastases due to *DLGAP5* overexpression, than in patients who are already suffering from systemic disease. The same effect, albeit weaker, was observed concerning the nodal status.

In several molecular CRC subtypes such as the low EphB2-ISC subgroup, the low crypt-like subtype, and stage A–like subtype, overexpression of *DLGAP5* is also associated with worse overall survival. Furthermore, *DLGAP5* overexpression correlates with worse prognosis in stage II or III CRC with high recurrence risk defined by the Oncotype DX assay. These results taken together suggest the importance of *DLGAP5* in defining a more aggressive cancer phenotype.

Interestingly, we noticed that *DLGAP5* overexpression correlates with poor prognosis in well-differentiated CRC. On the other hand, in CRC with a stem cell gene signature, a *DLGAP5* overexpression correlates with better prognosis. This could be related to the multiple regulatory pathways involved in cell spindle assembly and chromosome segregation. In addition, other molecular mechanisms may predominate in high grade or stem cell–like tumors, whereas in less deregulated tumors *DLGAP5* overexpression leads to measurable prognostic effects.

We observed that an overexpression of *DLGAP5* has an opposite effect on survival in the Budinska subtypes A (surface crypt-like, *DLGAP5* is a positive prognostic factor) and B (low crypt like, *DLGAP5* is a negative prognostic factor). Subtype B highly expresses canonical Wnt signaling target signatures. Subtypes A and normal samples showed instead a low expression of these signatures [22]. Recently, the interplay between Aurora A kinase, Wnt, and RAS-MAPK signaling in various cancers has been described [33]. Interestingly, Aurora A kinase has been shown to enhance Wnt and Ras-MAPK signaling. Aurora A kinase is encoded by *AURKA*, which is frequently amplified in colorectal cancer [34]. The interconnected regulation of Aurora A and DLG7 has been previously described [35–37]. All this could explain the opposite effects of *DLGAP5* expression in subtype A and subtype B CRC. In particular, in subtype B (high expression of canonical Wnt signaling target signatures), *DLGAP5* may play a role in the interaction between the Aurora A and the Wnt pathways, thus leading to a more aggressive tumor phenotype.

In conclusion, our results corroborate the hypothesis that DLG7/*DLGAP5* represents a potential biomarker in CRC with differential effects on molecular subtypes of CRC. Although there is sufficient evidence pointing towards DLG7/*DLGAP5* as a negative prognostic marker for most patients, its differential effects in some molecular subtypes require more experiments in order to fully understand the role of DLG7/*DLGAP5* in CRC.

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Author contribution list

1. Vittorio Branchi: study design and supervision, data collection, statistical analysis, data interpretation, drafting of the manuscript
2. Sebastian Garcia: data collection, statistical analysis, data interpretation, critical revision of the manuscript
3. Praveen Radhakrishnan: data interpretation, critical revision of the manuscript
4. Balázs Győrffy: statistical analysis, critical revision of the manuscript
5. Barbara Hissa: data interpretation, critical revision of the manuscript
6. Martin Schneider: data interpretation, critical revision of the manuscript
7. Christoph Reissfelder: data interpretation, critical revision of the manuscript
8. Sebastian Schölch: study design and supervision, statistical analysis, data interpretation, drafting of the manuscript

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Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (ethics committee of the University of Heidelberg, ethics committee number 323/2004) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent was obtained from all individual participants included in the study.

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