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ORIGINAL ARTICLE

Expression of the chemokine decoy receptor D6 is decreased in colon adenocarcinomas

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Abstract Recruitment of immune cells to tumors is a complex process crucial for both inflammation-driven tumor progression and specific anti-tumor cytotoxicity. Chemokines control the directed migration of immune cells, and their actions are partly controlled by nonsignaling chemokine decoy receptors. The role of the receptors such as D6, Duffy antigen receptor for chemokines and ChemoCentryx chemokine receptor in immunity to tumors is still unclear. Using real-time PCR, we detected significantly decreased expression of D6 mRNA in colon tumors compared to unaffected mucosa. D6 protein was expressed by lymphatic endothelium and mononuclear cells in the colon lamina propria and detected by immunohistochemistry in two out of six tissue samples containing high D6 mRNA levels, whereas no staining was observed in any tissue samples expressing low mRNA levels. When examining the density of lymphatic vessels in colon tumors, we detected a marked increase in vessels identified by the lymphatic endothelial marker Lyve-1, excluding passive regulation of D6 due to decreased lymphatic vessel density. In parallel, the Treg-recruiting chemokine CCL22, which is sequestered by D6, was threefold increased in tumor tissue. Furthermore, we could show that low D6 expression correlated to more invasive tumors and that tumor location influences D6 expression, which is lower in the more distal parts of the colon. The data support that regulation of D6 by colon tumors results in altered levels of proinflammatory CC chemokines, thereby shaping the local chemokine network to favor tumor survival. This may have implications for the design of future immunotherapy for colon cancer.

Keywords Colon adenocarcinoma · Chemokine receptor · D6 · DARC · CCX-CKR

Introduction

Tumors are commonly poorly immunogenic, and activated tumor-specific lymphocytes often fail to infiltrate the tumor, challenging the effectiveness of immunotherapy as treatment. Instead, the tumor microenvironment evidently attracts immune cells beneficial for tumor survival [1]. Chemokines must be considered in immunotherapy strategies as they control the migration of leukocytes, during both homeostatic and inflammatory conditions. Chemokines are divided into four subgroups depending on the position of the first two cysteine residues relative to the NH_2 terminus, defined as C, CC, CXC and CX_3C chemokines [2, 3]. During tumor growth, several inflammatory chemokines are produced by both tumor and stromal cells in response to hypoxia, leading to recruitment of selected leukocytes to the tumor. Chemokines produced in the tumor microenvironment may also contribute directly to cancer progression by promoting angiogenesis and metastasis [4–6].

Chemokine receptors are seven transmembrane-spanning receptors, which are typically coupled to G-proteins and restricted to interact with either CC or CXC chemokines, all

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triggering cell movement [7]. Chemokine decoy receptors, on the other hand, bind and internalize chemokines, but are unable to induce intracellular signaling and cell movement in response to chemokine binding due to mutations in the DRYLAIV motif, preventing G-protein coupling and subsequent intracellular signaling. Decoy receptors also bind to a wider range of chemokines than signaling receptors [8]. The chemokine decoy receptors comprise D6, Duffy antigen receptor for chemokines (DARC) and ChemoCentryx chemokine receptor (CCX-CKR) [9–12]. D6 specifically binds inflammatory CC chemokines, while DARC has affinity for both CC and CXC chemokines and is the only mammalian chemokine receptor to bind ligands from more than one chemokine subfamily [12, 13].

The proposed function of D6 is to sequester inflammatory chemokines, thereby limiting inflammation and facilitating resolution [14–16]. Recently, D6 has also been shown to influence entry of antigen-presenting cells from inflamed peripheral tissue into lymph nodes. Mice that lack D6 accumulate inflammatory CC chemokines and inflammatory leukocytes outside lymph nodes, apparently impairing lymph node entry of CCR7-expressing antigen-presenting cells and leading to their retention in inflamed tissue [17]. D6 is expressed by a variety of tissues including placenta, skin, leukocytes, lung and gut, mainly by lymphatic endothelial cells but also by immune cells [18–20]. DARC is expressed on red blood cells and is hypothesized to act as a sink for excessive circulating chemokines [21, 22]. DARC also transports chemokines across blood endothelial cells, exposing them to passing leukocytes in the blood and thereby promoting adhesion and transendothelial migration [22–25]. CCX-CKR, expressed in many tissues including intestine and lung, binds the homeostatic chemokines, CCL19, 21 and 25, which are internalized and targeted for intracellular degradation [10, 26]. Murine studies suggest that CCX-CKR functions in regulating chemokine levels around draining lymph nodes, as defective CCX-CKR function results in elevated chemokine levels and decreased cell numbers in lymph nodes in both homeostatic and inflammatory conditions [27, 28]. Experimental studies have shown that DARC expression has a negative impact on tumors in lung, breast and prostate by inhibiting tumor metastasis and angiogenesis [29, 30]. CCX-CKR overexpression in human breast cancer cells inhibits their proliferation and invasion of Matrigel in vitro, and also tumor growth and metastasis in vivo. In parallel, low CCX-CKR expression is correlated with lymph node metastasis and poor survival in patients with breast cancer [31]. Similarly, when overexpressed in breast cancer cells, D6 attenuates proliferation and invasiveness in vitro and in vivo in murine systems. In accordance with this, D6 expression in human breast cancer is negatively correlated to both lymph node metastasis and clinical tumor stage [32]. More recent studies on human breast and gastric cancer tissues have also indicated that a combined expression of DARC, D6 and CCX-CKR is a prognostic marker for relapse-free survival as well as for overall survival. It was also reported that co-expression of the chemokine decoy receptors is much lower in invasive compared to noninvasive breast carcinoma and healthy breast tissues [33, 34].

The role of chemokine decoy receptors in intestinal inflammation and carcinogenesis remains unclear. Bordon et al. [35] showed that D6-deficient mice are less susceptible to DSS-induced colitis compared to wild-type controls. Even so, inflammatory cytokines were increased in the colon of D6-deficient mice, and IL-17A was shown to protect the D6-deficient mice from colitis. In contrast, Vetrano et al. [36] reported that D6-deficient mice had increased susceptibility to DSS-induced colitis and tumor formation. However, the same report revealed elevated D6 expression in human IBD and IBD-associated colon cancer compared to healthy tissue.

Based on the chemokine scavenging activity of the decoy receptors, we hypothesized that regulation of their expression may contribute to development and progression of spontaneous colon tumors. In the present study, we investigated the expression of D6, DARC and CCX-CKR in human colon adenocarcinoma and unaffected colon. We report that D6 expression is strongly decreased in colon tumors compared to unaffected tissue from the same individual. Furthermore, D6 expression was lower in more advanced tumors, and our data indicate that D6 down-regulation could be a mechanism used by tumors to shape the local chemokine network to favor tumor survival.

Materials and methods

Volunteers and specimen collection

Forty-one patients undergoing partial colectomy at Sahlgrenska University hospital were included in this study (Table 1). The study was approved by the local regional board of ethics in medical research in west Sweden and therefore has been performed according to the Declaration of Helsinki 1964 ethical standards and its later amendments. Informed consent was obtained from each patient before participation. All patients had histologically verified adenocarcinoma and no history of autoimmune disease, or radiotherapy or chemotherapy 3 years prior to colectomy. Information on tumor location, differentiation grade, tumor stage, lymph node spread and distant metastases was obtained from the pathology report. During or immediately after colectomy, a strip of tumor mucosa was collected together with unaffected tissue. Hence, all analyses were performed on paired tumor and unaffected mucosa samples. Mucosa was considered unaffected if collected at least 5 cm away from the tumor, and the majority of samples

Table 1 Characteristics of the colon cancer patients included in the study

	Females	Males
n	18	23
Age	41–95	53–89
Tumor location		
Cecum	6	5
Ascending	4	4
Transverse	5	2
Descending	–	1
Sigmoid	6	8
Differentiation grade		
High	–	–
Medium	14	18
Low	3	4
Mucinous	2	–
Tumor stage		
T1	–	2
T2	3	1
T3	12	15
T4	4	4
Lymph node spread	8	10
Distant metastases	–	4

were also histologically examined. Small pieces of tissue were snap-frozen for protein extraction, incubated in RNA later for RNA isolation or embedded in OCT for immunohistochemistry or immunofluorescence.

Quantitative real-time PCR

Tissue biopsies from both unaffected and tumor mucosa were incubated in RNAlater (Ambion) over night at 4 °C with subsequent storage at −80 °C until use. Frozen biopsies were lysed and homogenized (TissuelyserII, Qiagen) before total RNA was isolated using the RNeasy mini kit (Qiagen). RNA integrity was confirmed by running RNA samples on 1 % agarose gels, and RNA concentration was determined spectrophotometrically (Nano drop ND-100, software version 2.5.1). Samples were DNase treated (DNA free, Ambion) to erase any contaminating DNA. The Omniscript kit (Qiagen) was used for cDNA synthesis, using 500 ng RNA as template in a total reaction volume of 20 µl. Each real-time PCR mixture contained 40 ng cDNA, 1× Taqman Universal PCR Master Mix, 1 µl Taqman assay mix, containing forward and reverse primer and a Taqman probe (D6: Hs00174299_m1, DARC: Hs01011079_s1, CCX-CKR: Hs00356608_g1, 18S: rRNA:Hs99999901_s1, CCL22: Hs99999075_m1), and ribonuclease-free water, at a final volume of 20 µl. All reagents were purchased from Applied Biosystems. Assays were run at standard thermal cycling conditions described for the 7500 real-time PCR system (Applied Biosystems). The comparative CT method was used for gene expression analysis, in which 18 s served as endogenous control [37]. To confirm specificity of primers and probe, real-time PCR products were run on 2 % agarose gels and stained with ethidium bromide.

Immunohistochemistry and immunofluorescence

Tissue sections (8 µm) were cut onto glass slides and fixed in ice-cold acetone before storage at −80 °C. Sections were rehydrated in cold PBS following blocking of endogenous biotin with an avidin/biotin kit (Molecular probes), according to manufacturer's instructions. For immunofluorescent detection, sections were incubated in PBS with polyclonal goat anti-human DARC (Everest Biotech) for 1 h followed by incubation with a biotinylated secondary donkey anti-goat (Jackson Immuno Research) antibody for 1 h. Then, the same sections were incubated for 30 min with mouse anti-human von Willebrand factor (DAKO) for 30 min before incubation with a DyLight 549-conjugated donkey anti-mouse secondary antibody (Jackson Immuno Research) for 30 min. Finally, sections were incubated with an Alexa Fluor® 488-conjugated streptavidin for 30 min. All incubations were performed at room temperature. Sections were mounted with ProLong® Gold (Molecular Probes). For immunohistochemical detection, sections were incubated with polyclonal goat anti-human LYVE-1 (R&D systems) or polyclonal rabbit anti-D6 (Sigma) overnight at 4 °C, followed by incubation with a biotinylated rabbit anti-goat or goat anti-rabbit secondary antibody (DAKO) for 30 and 45 min, respectively, at room temperature. For detection, sections were incubated with streptavidin-HRP (ABC-Elite, Vectastain) for 45 min before incubation with DAB (Histolab). Sections were counterstained in hematoxylin and mounted with Mountex (Histolab). Suitable isotype control antibody was used in parallel in all experiments. Images were acquired using the Axiovision 4.7.2 (Carl Zeiss) or Zen 2012 (Carl Zeiss) and analyzed in Biopix 2.0.21 (Biopix).

Protein extraction and detection of chemokines

Biopsies from tumor and unaffected mucosa from each patient were incubated in 600 µl PBS containing 2 % saponin, 100 µg/ml soybean trypsin inhibitor (Sigma), 350 µg/ml pefabloc and 0.1 % bovine serum albumin overnight at 4 °C. Each suspension was centrifuged at 13,000×*g* for 5 min, and supernatants were collected and used for the detection of chemokines. Concentrations of CCL8 (MCP-2) and CCL22 (MDC) were determined using Duoset ELISA (R&D systems) according to manufacturer's instructions. CCL2 (MCP-1) and CCL5 (RANTES) were detected using the cytometric bead array chemokine analysis (Becton–Dickinson), according to manufacturer's instructions. The

chemokine concentrations in protein extracts were normalized to total protein concentrations in the extracts as measured by using the BCA protein assay (Thermo Fisher Scientific) following desalting on Zeba™ micro desalt spin columns (Thermo Fisher Scientific).

Statistical analysis

All statistical analyses were performed in SPSS 14.0 or PRISM 5, using the nonparametric Wilcoxon signed-rank test or paired *t* test when $n < 10$. p values <0.05 were considered statistically significant.

Results

Expression of chemokine decoy receptor D6 mRNA is decreased in colon adenocarcinoma

To determine the expression of chemokine decoy receptors, total RNA was extracted from colon adenocarcinomas and unaffected mucosa from patients undergoing colectomy, and mRNA expression was measured using real-time PCR. We detected a substantial and significant decrease in D6 mRNA expression in tumors compared to unaffected mucosa ($p < 0.001$, median decrease was 15-fold) (Fig. 1a). In contrast, similar levels of DARC and CCX-CKR mRNA were detected in tumor and unaffected mucosa (Fig. 1c, d), suggesting a specific down-regulation of the chemokine decoy receptor D6 in tumors. The initial analysis of 13 patients was then expanded to enable comparisons between D6 expression and tumor and patient characteristics. Generally, expression levels of DARC (median Ct-value 28) and CCX-CKR (median Ct-value 29.3) in the unaffected colon mucosa were higher than the expression level of D6 (median Ct-value 35.7). Analysis of real-time PCR products on 2 % agarose gels confirmed specificity of the real-time PCR assays as only single products were detected with ethidium bromide (data not shown). To determine whether D6 mRNA and protein expression correlates, D6 expression was also detected by immunohistochemistry in six tissue samples expressing high levels of D6 mRNA and six samples with low D6 mRNA expression, all from unaffected mucosa as D6 expression in tumors is low. D6 protein was detected on vessel-like structures as well as on mononuclear cells in the lamina propria in two out of six samples with high D6 mRNA levels, but could not be detected in any of the samples where D6 mRNA was low (Fig. 1b). Further, staining with Lyve-1 identified D6-positive vessels as lymphatic endothelium in consecutive tissue sections (data not shown).

The density of lymph vessels is elevated in colon adenocarcinoma

D6 and DARC are strongly expressed in lymphatic and blood vessel endothelial cells, respectively [20, 38]. As

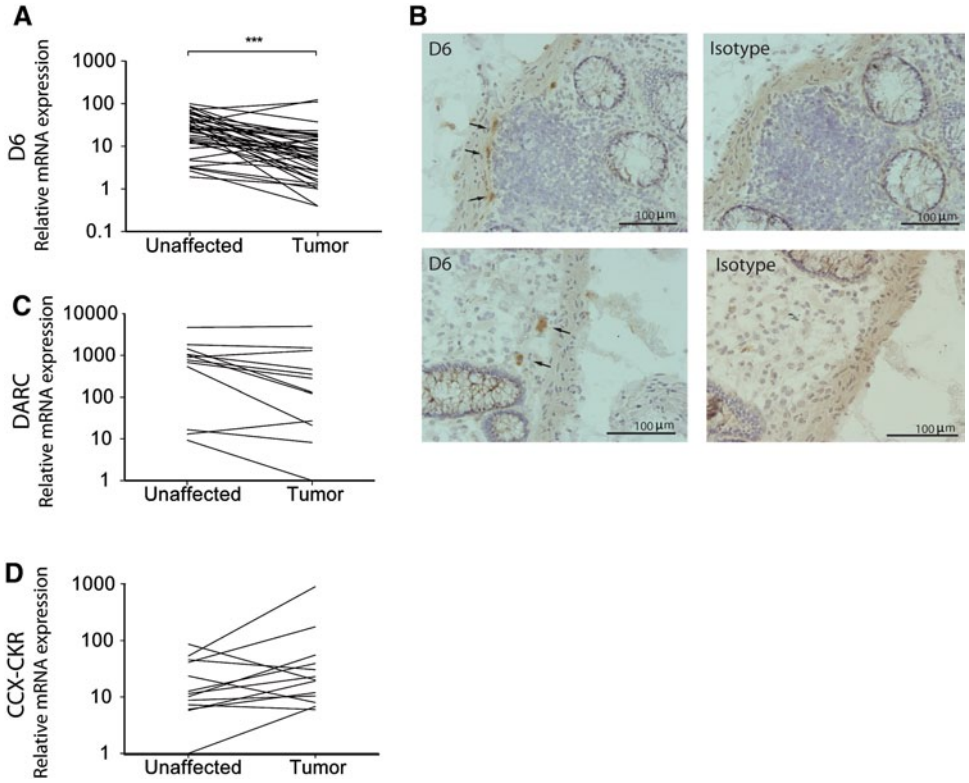

Fig. 1 The expression of D6 mRNA is decreased in colon tumor mucosa compared to unaffected. **a** D6 mRNA was examined in paired tumor and unaffected colon mucosa samples, using real-time PCR. Values were calculated using the ΔΔCt method and shown as fold change relative to a calibrator sample, using 18 s as endogenous control ($n = 40$). **b** Immunohistochemical detection of D6 (*left panel*, indicated by *arrows*) on vessel-like structures (*upper panel*) and single cells (*lower panel*) in unaffected lamina propria. Isotype control is shown in the right panel. **c** DARC mRNA expression ($n = 12$). **d** CCX-CKR mRNA expression ($n = 13$). Graphs show paired values from the same individual. ***$p < 0.001$

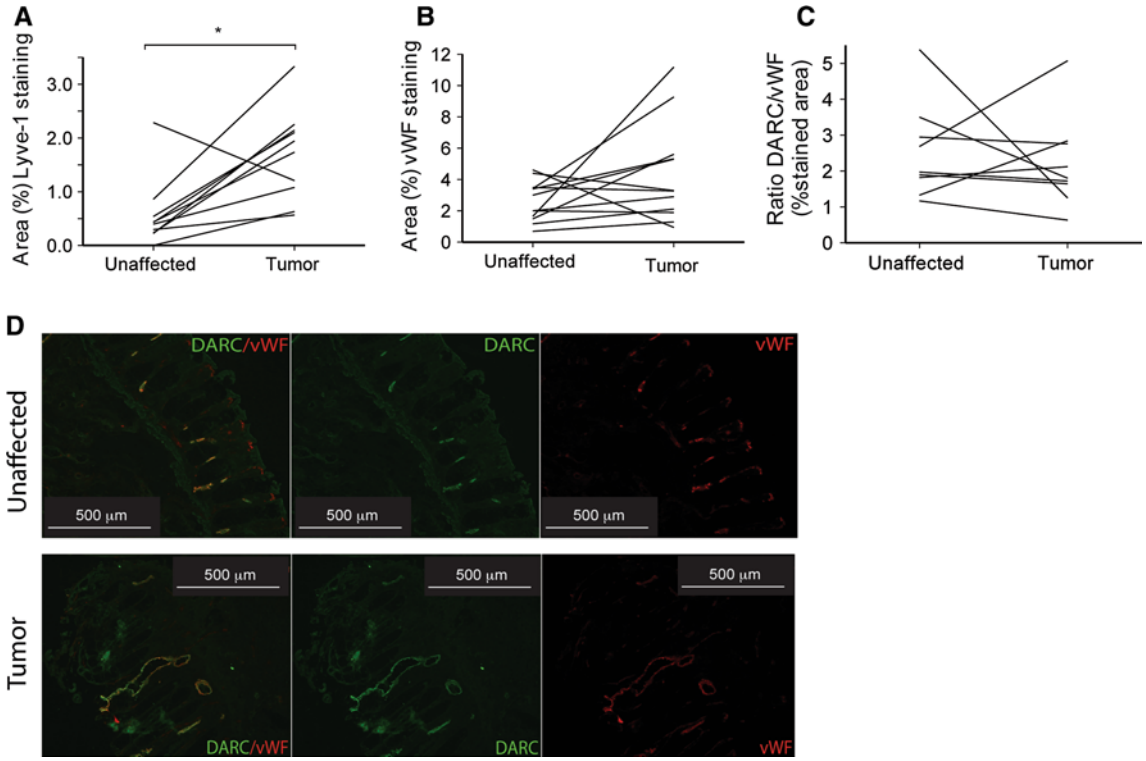

Fig. 2 Lymph and blood vessel density in tumor and unaffected colon mucosa. **a** The lymph endothelial marker Lyve-1 was analyzed on tumor and unaffected colon tissue sections using immunohistochemistry ($n = 10$). **b** Immunofluorescent detection of vWF in colon tumor and unaffected tissue sections ($n = 12$). **c** DARC and vWF were detected by double immunofluorescent staining, and DARC staining was normalized to vWF ($n = 9$). **d** Representative immunofluorescent staining of DARC (*green*) and vWF (*red*) in unaffected colon mucosa (*upper*) and colon tumor (*lower*). Graphs show paired values from the same individual presented as percentage stained area of total tissue section area. *$p < 0.05$

angiogenesis is a common feature in colon tumors, we examined the possibility that our results reflected changes in lymph or blood vessel density rather than regulation of chemokine decoy receptors in the tumor microenvironment. Decreased D6 expression in tumor tissue could not be explained by a decrease in lymph vessel density, as immunohistochemical staining detected increased expression of the lymphatic endothelial marker LYVE-1 in nine out of 10 tumors examined (Fig. 2a). Thus, if lymph endothelial cells are a major source of D6 in the colon, the decreased expression that we observed in the tumors is even more pronounced on a per-cell basis. Similarly, an altered blood vessel density in tumors compared to unaffected mucosa could conceal alterations in DARC expression, so this was examined in both tumor and unaffected mucosa. Double immunofluorescent staining with DARC and the blood endothelial marker von Willebrand factor (vWF) demonstrated that blood vessel density was elevated in some tumors compared to unaffected mucosa, but was not significantly different within the study population (Fig. 2b). Further, when related to vWF, DARC protein expression was not altered in tumor compared to unaffected colon mucosa, confirming our real-time PCR data (Fig. 2c and d).

D6 expression correlates with patient and tumor characteristics

As decreased D6 expression was a prominent feature of most colon tumors in our patient material, but not all, we investigated possible correlations between strongly decreased D6 in the tumor and tumor characteristics. Intestinal D6 expression was not dependent on age or sex of the patient (data not shown). As shown in Fig. 1, D6 expression in unaffected mucosa varied considerably between individuals, and to compensate for this variation, we used the difference in D6 expression between unaffected and tumor mucosa for each paired sample, when relating D6 expression to different tumor parameters. We were unable to find any relationship between D6 expression and lymph node spread or distant metastasis (data not shown). However, D6 expression was correlated to invasiveness, as it was significantly lower in T3 and T4 grade tumors compared to T1 and T2 ($p < 0.05$). The more invasive the

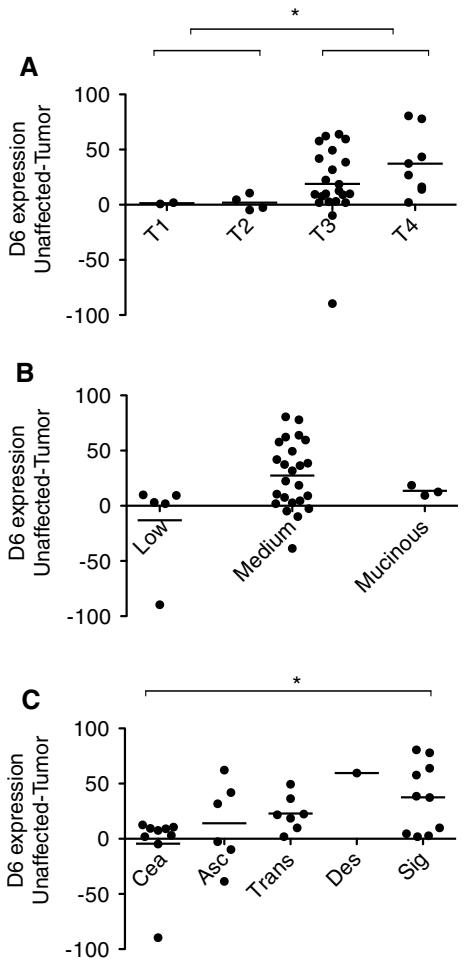

Fig. 3 Correlation of unaffected–tumor D6 expression with tumor characteristics. The difference in D6 mRNA expression between unaffected tissue and tumor tissue was compared to tumor invasiveness (**a**), differentiation (**b**) and location within the colon (**c**). Graphs show individual values from 29 to 34 patients. *$p < 0.05$

tumor, the less D6 was expressed (Fig. 3a). Tumor type did not correlate significantly to D6 expression even though there was a trend of medium differentiated tumors having lower expression of D6 than both less differentiated tumors and mucus-producing tumors (Fig. 3b). Interestingly, D6 expression was also significantly lower in tumors from the sigmoid part of the colon compared to the cecum, suggesting that D6 expression decreases in more distal parts of the colon (Fig. 3c).

The level of chemokine CCL22 is increased in colon adenocarcinoma

Finally, we investigated whether the decreased levels of D6 expression in tumors were accompanied with altered CC chemokine levels in the tumor mucosa. We used protein extracts from tumor and unaffected mucosa to determine the levels of selected chemokines (CCL2, CCL5, CCL8 and CCL22) sequestered by D6. Concentrations of CCL5 and CCL8 were not different between the unaffected mucosa and tumor (Table 2). On the other hand, CCL22 was present in significantly higher concentrations in tumor tissue compared to unaffected mucosa ($p < 0.01$) (Fig. 4a). CCL2 mRNA was also quantified, and results show increased levels ($p < 0.05$) of tumors compared to unaffected mucosa in seven out of 10 individuals (Fig. 4b). Also, CCL2 that has high affinity for D6 was lower in some tumors compared to unaffected tissue, but this difference did not reach statistical significance (Table 2). Despite the great redundancy of the chemokine system, these results indicate that lower D6 expression in tumors could lead to impaired sequestering of specific chemokines.

Table 2 Chemokine concentrations in tumor and unaffected tissues

	Tumor	Unaffected tissue
CCL2 (MCP-1)	148 ± 232	61 ± 48[a]
CCL5 (RANTES)	131 ± 165	119 ± 72
CCL8 (MCP-2)	132 ± 146	218 ± 457

[a] Chemokine concentration in pg/mg total protein (mean ± standard deviation)

Discussion

The present study shows that D6 expression is substantially decreased in colon adenocarcinomas compared to unaffected colon mucosa. In contrast, no difference in DARC and CCX-CKR mRNA expression was observed between tumor and unaffected mucosa. Previous work shows that D6 and DARC are strongly expressed in lymphatic and blood endothelial cells, respectively [20, 39]. D6 is also reported to be expressed by human monocytes, dendritic cells and B cells [19]. Our immunohistochemical staining indicates that D6 expression in the colon is found on lymphatic endothelial cells, but also on mononuclear cells in the lamina propria, thus confirming a previous study by Vetrano et al. [36]. Angiogenesis is frequently reported in tumors and could passively alter chemokine decoy receptor levels. Therefore, we examined vessel density in colon mucosa and found that blood vessel density was similar between tumor and unaffected mucosa, whereas lymph vessel density was clearly increased in the majority of tumor samples. Thus, the observed decrease in D6 expression in colon tumors is not simply a reflection of lymph vessel density, but instead, D6 appears to be actively regulated in the tumor microenvironment.

The notion that D6 could suppress malignant transformation and tumor progression was first suggested in a

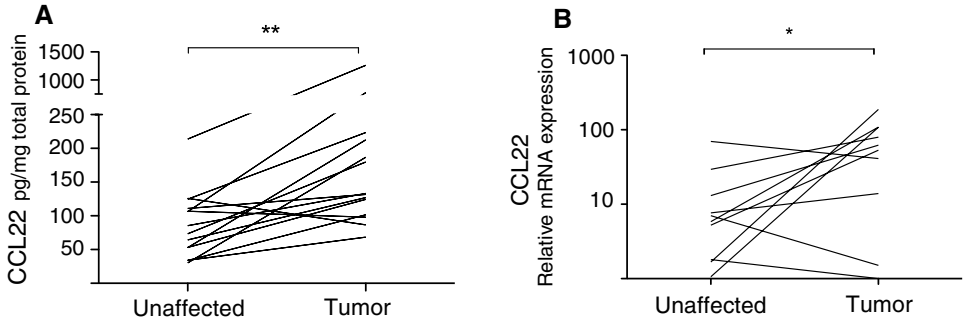

Fig. 4 The expression of CCL22 is elevated in colon tumor tissue. **a** CCL22 protein was determined in paired tumor and unaffected colon mucosa samples ($n = 10$) by Duoset ELISA and expressed as pg per mg total protein. **b** CCL22 mRNA was examined using real-time PCR. Values were calculated using the ΔΔCt method and shown as fold change relative to a calibrator sample, using 18 s as endogenous control. Graphs show paired values from the same individual. $*p < 0.05$, $**p < 0.01$

study demonstrating that $D6^{-/-}$ mice are highly susceptible to tumor formation in the skin following exposure to the irritant 12-O-tetradecanoyl phorbol-13-acetate compared to wild-type mice [40], implying that the absence of D6 creates an environment suitable for tumor growth. More recently, it was shown that D6 expression is positively correlated to increased disease-free survival in patients with breast cancer [32], further suggesting a role for D6 in controlling tumor progression. In contrast, a study of D6 expression in IBD-associated colon cancer indicated that D6 expression is increased in the tumor compared to unaffected tissue [36]. These results are contradictory to the present study, which shows that D6 expression is substantially and significantly decreased in colon tumor tissue. The different results may be explained by the fact that the patients examined in the current study do not have a history of IBD or mucosal inflammation, making it difficult to compare the two studies. However, our finding that the more advanced T3- and T4-type tumors had a lower expression of D6 than T1 and T2 tumors indicates that a relative lack of D6 contributes to tumor progression and that D6 may be an important part of anti-tumor immunity. The observation that D6-deficient mice are more susceptible to chemically induced colon carcinogenesis than wild-type mice further suggests a protective role for D6 against intestinal tumors [36].

A possible consequence of decreased D6 in the tissue would be elevated levels of D6-binding chemokines, as previously described [41]. Here, we show that the concentration of CCL22 and CCL2, normally sequestered by D6 with high affinity, was elevated in tumor tissue. Most remarkable was the significantly increased level of CCL22 protein in tumor compared to unaffected tissue. Our data indicate that the increased level of CCL22 in tumors is partly a consequence of increased production, as CCL22 mRNA was also elevated, possibly reflecting differences in immune cell infiltration between the tissues. The local availability of these chemokines would be dependent on the production rate, the natural breakdown and the scavenging ability of D6. Studies of chemokine levels in lung lavage from allergically challenged mice indicate that D6 is only able to regulate chemokine availability over a certain range of concentrations, and this may be the case also in colon tissue [42], potentially explaining why not all D6-binding chemokines are increased in the tumors. CCL22 is derived from macrophages, including tumor-associated macrophages [43], and attracts various cell types expressing CCR4. We have previously reported increased recruitment of $CCR4^+$ Tregs and $CD4^+$ conventional T cells to colon tumors [44], and similar findings are reported for other types of cancer as well [45]. In particular, the accumulation of $CCR4^+$ Tregs in several types of malignancies is shown to correlate strongly to CCL22 expression in the local tumor environment [46–49]. Some human colon cancer cell lines secrete CCL22 in vitro, but it is not clear whether this is true in vivo [50]. However, increased production of CCL22 and simultaneous suppression of D6 expression by colon tumors would be a sophisticated strategy to recruit immunosuppressive Tregs in order to escape anti-tumor immune responses. In addition, reduced D6 in the tumor may also result in impaired migration of antigen-presenting cells to lymph nodes, resulting in poor induction of immune responses to tumor antigens as suggested in a recent report from Lee et al. [17].

In conclusion, this study shows significantly decreased expression of the chemokine decoy receptor D6 in colon adenocarcinomas compared to unaffected colon mucosa. This suppression appears to be actively regulated as the density of lymph vessels—a major site of D6 expression in the gut—was significantly higher in tumors. Low D6 expression would lead to higher concentrations of chemokines, among them CCL22, and this would potentially contribute to the recruitment of Treg to colon tumors. As low expression of D6 correlated with more invasive

tumors, we suggest that decreased D6 expression is an immune evasion mechanism that tumors exploit to avoid adaptive immunity.

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

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