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Shift of syndecan-1 expression from epithelial to stromal cells during progression of solid tumours

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Abstract

Syndecan-1 (SDC-1), a protein found on cells and in the extracellular matrix, participates in cell proliferation, cell migration and cell–matrix interactions. SDC-1 expression correlates with the maintenance of epithelial morphology and inhibition of invasiveness. In the present study, a second SDC-1 mRNA isoform was identified and the expression of both transcripts was investigated in various normal and malignant tissues. Both transcripts were coexpressed at equal levels in all tissues and organs analysed. Cancer-profiling array (CPA) analysis of 241 non-enriched tumour and normal cDNAs revealed stronger upregulation of SDC-1 in tumour tissues as compared with oligonucleotide array-based expression analysis of SDC-1 in microdissected breast, prostate, lung, and colon carcinoma cells. With in situ hybridisation and immunohistochemistry it was demonstrated that this difference in SDC-1 expression originates from stromal cells present in tumour connective tissue. But only the cells in connective tissue surrounding breast, lung, colon and bladder carcinoma showed upregulation of SDC-1. These stromal cells were characterised as spindle cells with myofibroblastic differentiation and they may contribute to the dedifferentiation of tumour cells and the development of metastasis.

Keywords: Syndecan-1; Microdissection; Expression profiling; Tumour stroma

1. Introduction

The syndecans are transmembrane heparan sulphate proteoglycans expressed on the surface of many different adherent and non-adherent cells [1,2]. The family has four members, syndecans 1–4, which have diverse functions ranging from participation in cell–cell and cell–extracellular matrix adhesion to the regulation of heparan sulphate-binding growth factor activity as well as migration and proliferation [3–5]. All adherent cells express at least one syndecan but most express several [6].

Syndecan 1 (SDC-1), also named CD138, is expressed in a variety of cell types, including epithelial, endothelial and vascular smooth muscle cells. It consists of an extracellular 'syndecan domain' with amino acid residues capable of carrying heparan sulphate and chondroitin sulphate, a transmembrane domain and a cytoplasmic tail containing four conserved tyrosine and a conserved serine residue [7]. The SDC-1 ectodomain can be shed from the cell surface by proteolytic cleavage, usually at a juxtamembrane site [8]. SDC-1 is constitutively shed from cultured cells by proteolytic cleavage, but the SDC-1 concentration in the serum of healthy individuals is low [9]. The ectodomain shedding is controlled by a highly regulated mechanism [8,10,11].

SDC-1 expression as detected by immunohistochemistry (IHC) is associated with a poor grade of tumour differentiation. Loss of epithelial SDC-1 expression correlates with poor clinical outcome in patients with squamous cell carcinoma of the head and neck [12–14], mesothelioma [15], poorly differentiated non-small cell lung cancer [16,17] and in patients with hepatocellular carcinoma with high metastatic potential [18].

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Furthermore, in gastric cancer [19,20] and infiltrating breast carcinoma [21], the appearance of stromal expression of SDC-1 has been correlated with a devastating clinical course. In contrast to these observations, pancreatic adenocarcinoma over-expresses SDC-1 as compared to normal pancreas [22].

Thus, the role of SDC-1 in tumour growth may vary with tumour type and stage; decreased as well as increased expression of SDC-1 are recognised in human carcinomas. Here, we report on the expression analysis of SDC-1 RNA and protein in various human tumours and normal tissues.

2. Material and methods

2.1. Patients and tissue samples

Fifty-four prostate carcinomas, 26 breast carcinomas and their corresponding normal tissues were obtained from the Institute of Pathology, Charité University Hospital (Berlin, Germany). Fifty-eight colorectal carcinomas and matching normal samples were received from the Chirugische Klinik and Poliklinik I, University Hospital Benjamin Franklin (Berlin, Germany). Twenty-eight lung carcinomas and corresponding normal tissue samples were obtained from the Institute of Pathology, Charité University Hospital (Berlin, Germany) and Chirugische Klinik and Poliklinik I, University Hospital Benjamin Franklin (Berlin, Germany). Histopathological and clinical data were provided by each hospital. The freshly frozen tissue samples were subjected to laser-captured microdissection and Affymetrix DNA chip hybridisation experiments.

Formalin-fixed, paraffin-embedded breast, prostate, lung and colorectal carcinoma tissues were obtained from the Institute of Pathology, Charité University Hospital (Berlin, Germany), the Institute of Pathology, Reinhard-Nieter Hospital and St-Willehad Hospital (both Wilhelmshaven, Germany) and used in in situ hybridisation (ISH) and IHC. The commercial tissue-arrays MB1 and MBN1 were obtained from Biocat (Heidelberg, Germany). All tumour samples were classified according to histopathological criteria.

2.2. Microdissection

In brief, 30 serial frozen whole-mount 5- or 10-μm sections of tumour or normal tissues were air-dried and stained with haematoxylin. Every tenth slide was stained with haematoxylin/eosin for documentation. Suitable areas for microdissection were marked on these slides by a pathologist, using the supportive software of a microdissection device (Palm Microlaser Technologies, Bernried, Germany). The isolated tissues were pooled in guanidinium thiocyanate buffer (GTC) (PolyA Tract-Kit; Promega, Heidelberg, Germany) for RNA preparation.

2.3. RNA preparation and Affymetrix DNA chip hybridisation

The polyA+ RNA was prepared from the microdissected tissue with the PolyA Tract 1000 kit (Promega) according to the manufacturer's recommendations. On each sample, cDNA synthesis and repetitive in vitro transcription was performed three times according to Luo et al. [23], with minor modifications as described [24]. Hybridisation to the Affymetrix GeneChip Hu133 and detection of the labelled cRNA were performed according to Affymetrix instructions (Santa Clara, CA, www.affymetrix.com).

2.4. Bioinformatic analysis of expression data

Expression data were analysed with an algorithm developed at metaGen Pharmaceuticals (S. Röpcke, C. Pilarsky and T. Brümmendorf). In brief, for data normalisation the background was computed as the mean of the 2% darkest feature intensities; this background value was then subtracted from each feature value. Each feature value was then divided by the median of all feature values. The third quartile (75%) of the intensities from the 11 perfect match oligonucleotides was used as a representative expression value for each probe set. To distinguish signal from noise the Wilcoxon signed-rank test was applied to each probe set. A probe set was called detectable if the result of that test applied to its 11 probe pairs (perfect match vs. mismatch oligonucleotide) had a significance of $P < 0.05$.

The expression values were stored in a database and differential gene expression was determined as follows. An expression ratio was calculated for all probe sets that were identified as exhibiting differential gene expression according to the following rules. If expression was detectable in both the normal and the tumour sample according to the Wilcoxon test ($P < 0.05$) and tumour/normal (T/N) > 2.0 or T/N < 0.5, T/N was taken as the expression ratio. If expression was undetectable in either the normal or the tumour sample, the ratio T/N was computed to either T/N $= 2$ or T/N $= 0.5$. If expression was undetectable in both the normal and tumour sample ($P > 0.05$), no expression ratio was calculated. For each probe set we calculated, for all pairs of tumour and normal tissues, how often an expression ratio <0.5 (downregulated in the tumour) or >2 (upregulated in the tumour) was observed.

2.5. Northern blot analysis

As cDNA probes, two different fragments of human *SDC-1* were used: an *Eco*RI fragment that encodes the

first 1170 bp of the SDC-1 mRNA (GenBank Accession No. NM_002997) and a 586 bp, polymerase chain reaction-derived fragment containing the additional identified sequence of prolonged 3′ UTR (forward primer: 5′-gtgtgacgtgaccggacttttc-3′; reverse primer: 5′-cccacggaccagcagatgagca-3′). The complete mRNA sequence of 3149 bp was submitted to GenBank (Accession No. AJ551176).

The multiple tissue Northern blot (MTN) and the cancer profiling array (CPA) were purchased from BD Biosciences Clontech (Heidelberg, Germany) and hybridised using [α^{32}P]dCTP (Amersham, Braunschweig, Germany) labelled probes according to the manufacturer's recommendations. The CPA consists of SMART-amplified cDNA from 241 matched normal and tumour tissues from individual patients. Samples on the CPA are normalised by the provider to four housekeeping genes [25]. Detailed clinical information and a complete list of tissues can be found on the provider's website (www.clontech.com). After exposure to a Phosphoimager screen for 12 h, the blots were scanned with a Fujifilm BAS-Reader 1800-II at 50-μm resolution. The results were quantified using the AIDA software (raytest GmbH, Straubenhardt, Germany). A grid was applied to the scanned image of the dot blot to quantify the intensity of the hybridisation signal of each spot. After background subtraction, the image was normalised using all spots on the membrane as reference points. The threshold values for up- and downregulation were chosen based on T/N intensity ratios for all patient samples.

2.6. In situ hybridisation

The paraffin-embedded tissues sections were deparaffinised and rehydrated, fixed in 4% paraformaldehyde, washed twice in phosphate-buffered saline (PBS), and processed for the experiment according to the manufacturer's instruction in the DIG-RNA labelling kit (Roche Applied Science, Mannheim, Germany). To generate riboprobes the pT7T3-Pac plasmid containing the 1170 bp fragment of SDC-1 cDNA was linearised with *Not*I for the antisense and *Xho*I for the sense probe. The probes were digoxigenin-labelled using the DIG-RNA labelling kit (Roche Applied Science). Hybridised probes were detected using alkaline phosphatase-conjugated anti-DIG antibody (1:5000 v/v) and BM Purple as substrate (Roche Applied Science). After refixation, the sections were counterstained for 5 min with Kernecht Rot (Merck, Darmstadt, Germany) and examined by a pathologist.

2.7. Immunohistochemistry

Paraffin-embedded tissue sections (3–5 μm thick) were deparaffinised and rehydrated. Endogenous peroxidase activity was quenched with 3% H_2O_2. Antigen was retrieved by pretreating the slides with citrate buffer (pH 6) in a microwave oven for 30 min at 100 W. The slides were then cooled to room temperature and non-specific hybridisation was blocked with Protein Block (DAKO, Hamburg, Germany). The sections were incubated for 1 h at room temperature with a mouse monoclonal antibody directed against SDC-1, B-B4 (RDI Research Diagnostics, Flanders, NY), at a final concentration of 2 μg/ml. This SDC-1-specific antibody recognises the epitope LPEV in the extracellular domain of human SDC-1 [26]. The primary antibodies against vimentin (M7020, 1:150), pan-cytokeratin (PanCK; M0821, 1:100), Ki-67 (M7240, 1:100), and α-smooth muscle actin (M0625, 1:150) were obtained from DAKO and used according to the manufacturer's instructions. The slides were then rinsed once with PBS plus 0.05% (v/v) Tween 20 and once with PBS, and then incubated for 10 min with the appropriate secondary antibody (biotinylated Polylink antibody; Biocarta). After a further washing, streptavidin–horseradish peroxidase (Biocarta) was applied to the slides and incubated for another 10 min. 3-Amino-9-ethyl carbazole (AEC) chromogen substrate (Romulin AEC; Biocarta) was used for antibody detection. Sections were counterstained with haemalaum. The staining intensity and the percentage of positive cells were scored by pathological examination. Finally, the staining of tumour tissue in comparison to normal tissue was scored as none, less, equal or higher expression.

3. Results

3.1. Two coexpressed SDC-1 mRNA transcripts

The available expressed-sequence tags from the public domain and the Incyte LifeSeqGold library were assembled on the 2458 bp human SDC-1 mRNA (GenBank Accession No. NM_002997) (Fig. 1(A-a)) and thereby an additional mRNA transcript was detected (GenBank Accession No. AJ551176). The newly identified transcript has a size of 3148 bp and uses an alternative poly-adenylation signal. The variation between these two transcripts is restricted to the 3′ untranslated region (UTR) and the open reading frame (ORF) is unchanged (Fig. 1A-b). When the cDNA probe 'c' from the 5′ end of the SDC-1 mRNA was hybridised to MTN, two signals at approximately 2.4 and 3.4 kb were clearly detectable in placenta, moderate signals were visible in liver, kidney and skeletal muscle, and weak signals in colon and lung (Fig. 1B). The two transcripts showed no differential expression pattern in the organs analysed. Using the cDNA probe 'd' from the 3′ end (Fig. 1A-d), the same MTN was hybridised and revealed only one signal at 3.4 kb with a similar expression pattern (Fig. 1C), indicating that probe 'd' derived from the 3′ UTR detects only the longer SDC-1 transcript.

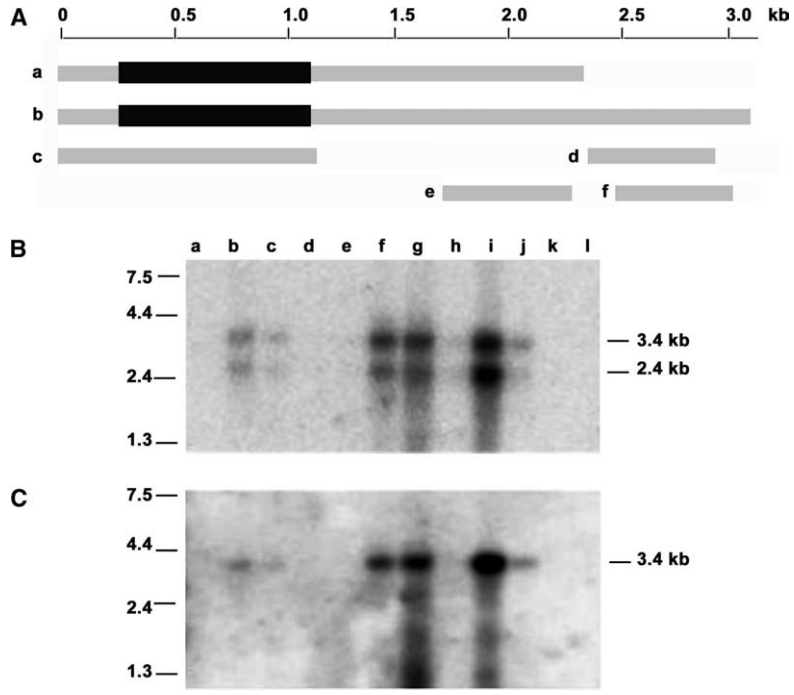

Fig. 1. SDC-1 expression detected on a human multiple tissue Northern blot (MTN). (A) The available expressed-sequence tags from the public domain as well as the Incyte LifeSeqGold library were assembled on the 2458 bp human SDC-1 mRNA (A-a) (GenBank Accession No. NM_002997) and an additional mRNA transcript of 3148 bp was detected (A-b) (GenBank Accession No. AJ551176). The variation between the two transcripts is restricted to the 3′ UTR and the open reading frames (indicated as black boxes) are unchanged. The cDNA probe 'c' (1170 bp) located at the 5′ end (A-c) and the cDNA probe 'd' (586 bp) specific for the newly identified transcript (A-d) were used in Northern blot analysis (see below). The grey boxes indicate the location of the SDC-1-specific probe sets 201287_s_at (A-e) and 201286_at (A-f), respectively, on the Affymetrix Hu133 array. (B) Northern blot analysis using cDNA probe 'c' on a MTN blot. The known 2.4 kb SDC-1 transcript and the newly identified 3.4 kb SDC-1 transcript are indicated. (C) Northern blot analysis using the cDNA probe 'd' for the newly identified transcript. The newly identified 3.4 kb SDC-1 transcript is indicated. *Abbreviations*: a, brain; b, heart; c, skeletal muscle; d, colon; e, thymus; f, spleen; g, kidney; h, liver; i, small intestine; j, placenta; k, lung; l, peripheral blood leucocytes.

3.2. Cancer-profiling array analysis of SDC-1 expression in matching tumour and normal tissue

The commercially available CPA, which contains 241 cDNAs from matching tumour and normal tissue and nine cDNAs from different human cell lines spotted on a nylon membrane, was hybridised with the cDNA probe 'c' (Fig. 1A-c) and exposed to a Phoshoimager screen (Fig. 2). SDC-1 was overexpressed in the majority of tumours. The highest differential expression of SDC-1 was found in breast (A), colon (C) and lung carcinomas (F), whereas the majority of renal cell carcinomas showed downregulation of SDC-1 in the tumour tissues (G). Quantification using a Phosphoimager (Table 1) confirmed that tumours derived from breast (37.7% of the analysed cases), uterus (38.6%), colon (15.4%), ovary (68.8%), lung (28.6%) and rectum (21.1%) showed upregulation of the SDC-1 transcript. In gastric carcinomas (D) no significant transcriptional difference was detectable, whereas in renal cell carcinomas (G) downregulation of SDC-1 was seen in 35% of the patients. Additionally, the nine human cancer cell lines (HeLa; Daudi; K562; HL-60; G361; A549; MOLT-4; SW480; Raji) all had weak SDC-1 expression. The negative controls on the CPA did not result in a hybridisation signal. Hybridisation of the CPA with the cDNA probe 'd' (Fig. 1A-d), which is specific for the newly identified transcript, showed the same expression pattern in all normal and tumour tissue samples (data not shown). The quantification confirmed the findings from the MTN blot (Fig. 1B and C), demonstrating that the relative levels of the two SDC-1 transcripts were maintained in normal and tumour tissues.

The CPA contains bulk cDNA amplified according to the SMART protocol [25]. Differential gene expression in stromal vs. epithelial cells cannot be detected when analysing complete tumour tissues. In contrast, analysis of RNA from microdissected tumour epithelium and normal epithelium allows a comparison of those genes that are differentially expressed in the normal and the malignant epithelial tissue.

3.3. SDC-1 expression in microdissected tumour and normal tissue measured by Affymetrix DNA chip analysis

Using laser-captured microdissection, normal epithelium and tumour cells from the same patient were separately enriched and collected from 26 breast, 28

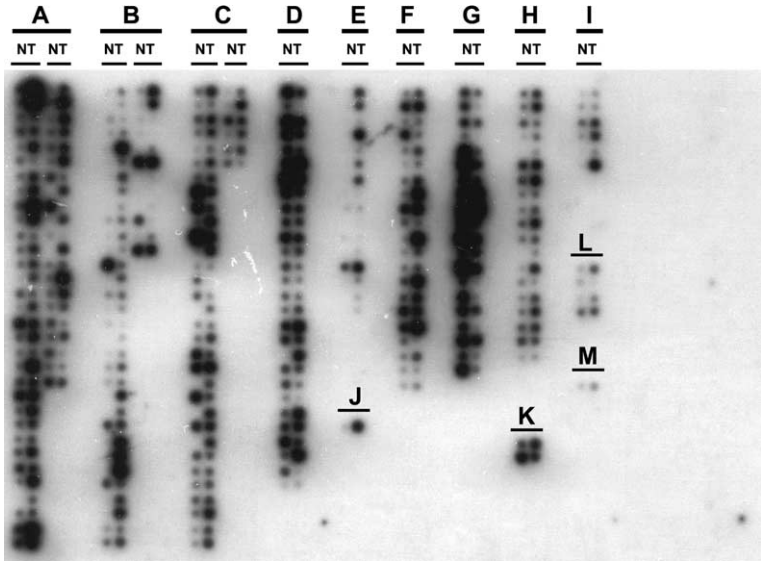

Fig. 2. Analysis of SDC-1 mRNA levels on the cancer profiling array (CPA). A CPA was hybridised with cDNA probe 'c' (A–C). SDC-1 mRNA was over-expressed in the majority of bulk tumours when compared to normal tissue from the same patient. A, breast; B, uterus; C, colon; D, stomach; E, ovary; F, lung; G, kidney; H, rectum; I, thyroid gland; J, cervix; K, small intestine; L, prostate; M, pancreas. N and T indicate the matching cDNA spots from SMART-amplified RNA from normal and tumour tissues, respectively. The nine human cancer cell lines (HeLa; Daudi; K562; HL-60; G361; A549; MOLT-4; SW480; Raji) all had weak SDC-1 expression. The negative controls on the CPA did not result in a hybridisation signal. For quantification using the Phosphoimager, see Table 2.

lung, 54 prostate, and 58 colon carcinoma samples, respectively. Isolated mRNA was amplified and hybridised to the Affymetrix Hu133 array. Two probe sets, 201286_at and 201287_s_at, interrogate SDC-1 mRNA on the Hu133 array. The expression values in Table 2 are derived from the probe set 201287_s_at that recognises the short and the long transcript (Fig. 1A-e). The absolute expression values and the ratio of SDC-1 expression between tumour and normal tissue were calculated for each patient. The summarised expression analysis is shown in Table 2. In six out of 26 breast carcinomas ($P = 6.7$) and six out of 28 lung carcinomas ($P = 0.0061$), upregulation of SDC-1 mRNA was detected within the tumour cell population, whereas no

Table 1
Quantification of the *SDC-1* signals on the cancer profiling array (CPA)

	Breast	Lung	Colon	Prostate	Stomach
n	53	21	39	4	27
All cases	1.56 ± 0.85	1.60 ± 0.76	1.22 ± 1.08	1.51 ± 1.24	0.87 ± 0.73
Ratio >2	20/53 (37.7%)	6/21 (28.6%)	6/39 (15.4%)	1/4 (25.0%)	2/27 (7.4%)
	2.48 ± 0.55	2.70 ± 0.55	3.11 ± 1.44	3.12	3.09 ± 1.32
Ratio 0.5–2	30/53 (56.6%)	14/21 (66.7%)	30/39 (76.9%)	2/4 (50.0%)	23/27 (85.2%)
	1.26 ± 0.39	1.38 ± 0.33	1.15 ± 0.40	1.51 ± 0.29	0.87 ± 0.39
Ratio <0.5	3/53 (5.7%)	1/21 (4.8%)	3/39 (7.7%)	1/4 (25.0%)	2/27 (7.4%)
	0.35 ± 0.05	0.37	0.47 ± 0.17	0.31	0.45 ± 0.06
	Ovary	Uterus	Kidney	Rectum	Thyroid gland
n	16	44	20	19	6
All cases	2.25 ± 1.50	1.64 ± 1.87	0.62 ± 0.25	1.39 ± 0.63	1.96 ± 1.20
Ratio >2	11/16 (68.8%)	17/44 (38.6)	0/20 (0%)	4/19 (21.1%)	3/6 (50.0%)
	2.82 ± 1.21	3.88 ± 1.94	–	2.32 ± 0.59	4.14 ± 3.13
Ratio 0.5–2	4/16 (25.0%)	25/44 (56.8%)	13/20 (65.0%)	15/19 (78.9%)	3/6 (50.0%)
	1.07 ± 0.49	0.89 ± 0.32	0.84 ± 0.16	1.32 ± 0.34	1.74 ± 0.48
Ratio <0.5	1/16 (6.3%)	2/44 (4.5%)	7/20 (35.0%)	0/19 (0%)	0/6 (0.0%)
	0.07	0.39 ± 0.01	0.34 ± 0.07	–	–

For analysis of SDC-1 mRNA expression on CPA, see Fig. 2.

The SDC-1 expression ratios are between non-enriched normal and tumour tissues from the same patient.

Ratios are displayed ±SD; differences considered significant at confidence levels greater than 95% ($P < 0.05$).

Tumours derived from breast (37.7% of the analysed cases) with a mean ratio of 2.48 ± 0.55, uterus (38.8%), colon (15.4%), ovary (68.8%), lung (28.6%) and rectum (21.1%) all show upregulation of SDC-1 mRNA.

Table 2
Differential expression of SDC-1 on microdissected tumour and normal tissues analysed on the Affymetrix Hu133 array

	Breast	Lung	Colon	Prostate
n	26	28	58	54
All cases	1.36 ± 0.55	1.43 ± 1.34	0.84 ± 0.67	0.80 ± 0.38
Ratio >2	6/26 (23.1%)	6/28 (21.4%)	4/58 (7.4%)	1/54 (1.9%)
	2.22 ± 0.31	3.39 ± 1.38	2.80 ± 1.08	2.11 ± 0.00
Ratio 0.5–2	20/26 (76.9%)	20/28 (71.4%)	46/58 (79.3%)	48/54 (88.8%)
	1.20 ± 0.3	1.27 ± 0.37	0.87 ± 0.27	0.89 ± 0.32
Ratio <0.5	0/26 (0.0%)	2/28 (7.1%)	8/58 (13.8%)	5/54 (7.4%)
	– ± –	0.42 ± 0.06	0.40 ± 0.08	0.40 ± 0.24

SDC-1 expression ratios between microdissected tumour cells and normal epithelial cells from the same patient; absolute cases and the mean ratio ± SD.

In 6 out of 26 breast tumours (23.1%) there is upregulation of SDC-1 mRNA with a mean ratio of 2.22 ± 0.31 in tumour vs. normal tissue.

21.4% of the lung carcinomas, there is weak upregulation compared to normal lung tissue. Colon and prostate samples reveal no significantly differential expression of SDC-1 in tumour and normal microdissected tissue.

significant upregulation was detected in 58 colon and 54 prostate carcinoma samples (Table 2). The probe set 201286_at, which recognises specifically the newly identified transcript (Fig. 1A-f), showed exactly the same distribution of expression ratios in microdissected tumour vs. normal tissues, albeit at a lower overall expression (data not shown).

Surprisingly, the Affymetrix array-based, RNA expression analysis of microdissected tumour and normal epithelial tissue revealed differential SDC-1 expression only in a minority of cases. This is in contrast to the results gained with RNA from complete tissues on the CPA.

3.4. SDC-1 expression in the tumour connective tissue

In order to clarify the discrepancy in differential SDC-1 expression between RNA from complete and microdissected tissue, the distribution of RNA and protein expression was analysed by ISH and IHC. First, the expression pattern of SDC-1 in normal tissue of breast, colon, pancreas, prostate, ovary, liver and lung was studied using the 1170 bp antisense riboprobe for ISH (see Fig. 1A–c). The epithelial cells showed expression of SDC-1 mRNA, but SDC-1 mRNA was not detected in the stromal cells that surround the normal epithelial cells in all tissue entities (data not shown).

The ISH of invasive breast carcinoma (Fig. 3(a)), colorectal adenocarcinoma (Fig. 3(b)) and invasive bladder carcinoma (Fig. 3(c)) with SDC-1 antisense riboprobe revealed SDC-1 expression in the stromal cells. This demonstrates that the stromal cells themselves express SDC-1 mRNA.

The expression pattern of SDC-1 in normal tissues was also studied by IHC staining on normal breast, colon, pancreas, prostate, ovarian and lung tissues using the monoclonal antibody B-B4 specific for SDC-1. The epithelial cells showed expression of SDC-1 protein, whereas it was not detected in the stroma that surrounds the normal epithelium (data not shown).

Two sets of results were obtained by IHC: tumour samples from 52 prostate and 18 pancreas carcinomas showed SDC-1 protein expression restricted to the tumour cells without staining of the connective stroma cells (data not shown), whereas IHC of 28 breast, 18 bladder, 18 colorectal and 19 lung carcinoma samples revealed strong staining of the connective stromal cells (see Fig. 3(d)–(f)). The tumour cells themselves showed a non-homogeneous SDC-1 expression pattern. Some tumour cells had reduced SDC-1 expression, whereas others revealed either no change or a slight upregulation of SDC-1 at the protein level. The breast carcinoma showed strong immunostaining of cells in the stromal compartment, whereas the epithelial tumour cells revealed no or only weak SDC-1 staining (Fig. 3(d)). In the majority of colorectal carcinomas, the stromal cells and the tumour cells showed diffuse cytoplasmic staining (Fig. 3(e)). The invasive bladder carcinoma samples displayed strong staining of the tumour cells and the stromal connective tissue compartments. In the latter, the signal decreased with increasing distance from the tumour cells (Fig. 3(f)).

3.5. SDC-1 is expressed in spindle cells with myofibroblastic differentiation

In a further analysis, we examined whether SDC-1-positive stromal cells are derived from tumour cells that had adopted a mesenchymal phenotype, or whether SDC-1 expression was induced in those cells of the connective tissue that are close to the tumour cells. IHC with antibodies directed against marker proteins indicative of defined stromal cell types [27] was performed on serial sections of breast carcinoma from five different patients; typical results are shown in Fig. 4. SDC-1-positive stromal cells (Fig. 4(a)) showed no staining for cytokeratins as demonstrated with an anti-PanCK antibody, which is a marker for glandular epithelial cells (Fig. 4(d)). Only the tumour epithelial cells were positive for both SDC-1 and CK. The tissue was also stained

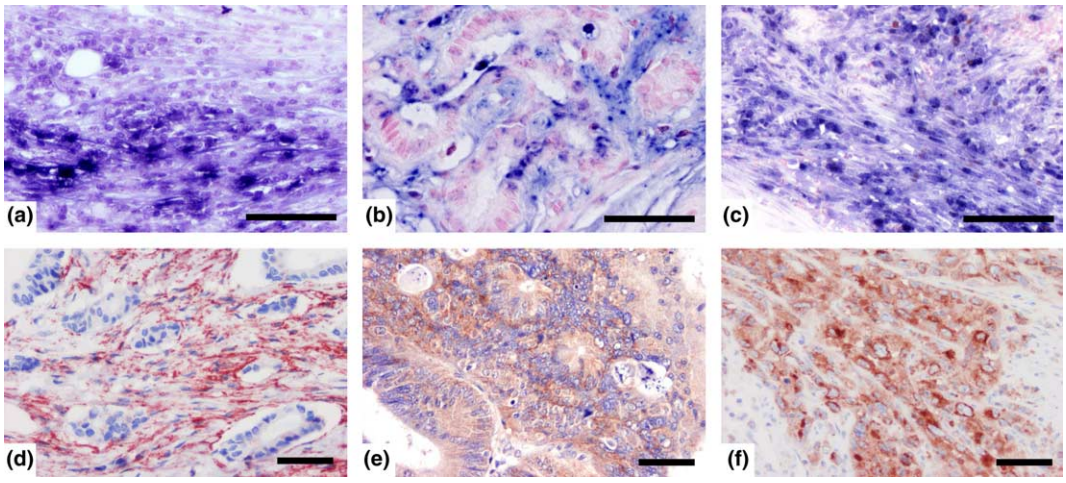

Fig. 3. Detection of SDC-1 expression in the stroma by in situ hybridisation (ISH) and immunohistochemistry (IHC). (a–c) ISH with the SDC-1 DIG-labelled antisense riboprobe, which detects both SDC-1 transcripts. SDC-1 mRNA expression in stromal cells detected in invasive breast carcinoma (a), in colorectal adenocarcinoma (b) and in invasive bladder carcinoma (c). A SDC-1 sense riboprobe did not reveal a staining signal. Invasive breast (d), colorectal (e), and invasive bladder carcinoma (f), respectively, were stained with the SDC-1-specific antibody B-B4. The breast carcinoma showed strong staining of the stromal compartment, whereas the epithelial tumour cells revealed no or only weak SDC-1 staining (d). In the majority of the colorectal carcinoma, the stromal and the tumour cells show a strong diffuse cytoplasmic staining (e). The invasive bladder carcinoma displays strong staining of tumour cells and stromal connective tissue, decreasing with increasing distance from the tumour cells (f). Scale bar = 50 μm.

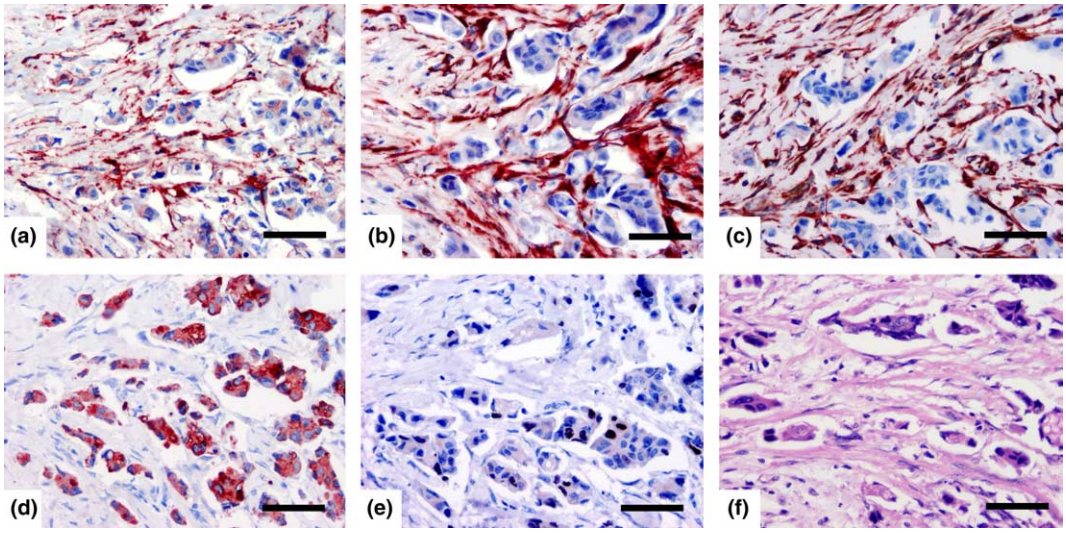

Fig. 4. Immunohistochemical analysis of SDC-1 expression in invasive ductal breast carcinoma. Serial sections of invasive ductal breast carcinoma stained with antibodies directed against SDC-1 (a), α-smooth muscle actin as marker for myoepithelial and muscle cells (b), vimentin as marker for fibroblasts (c), with a PanCK antibody against cytokeratins as marker for glandular epithelial cells (d), the proliferation marker Ki-67 (e), or with HE (f). Bound primary antibody detected with horseradish peroxidase-coupled secondary antibody, AEC staining and haemalum counterstain. Scale bar = 50 μm.

with an antibody directed against α-smooth muscle actin as a marker for myoepithelial, myofibroblast and smooth muscle cells, and a clear signal was detected in the SDC-1-positive stromal cells (Fig. 4(b)). Staining with an anti-vimentin antibody as marker for mesenchymal fibroblasts resulted in signals in the same cells as those that were positive for SDC-1 and for α-smooth muscle actin (Fig. 4(c)). Ki-67 staining was performed to detect proliferating cells (Fig. 4(e)). In summary, the IHC counterstaining experiments suggest that the SDC-1-positive stromal cells that surround the invasive ductal breast carcinoma cells are spindle cells with myofibroblastic differentiation.

4. Discussion

A number of studies have investigated the expression of SDC-1 in human carcinomas. Decreasing SDC-1 expression was correlated with advanced tumour stage

and an increased metastatic tendency of the cancer cells in a wide range of tumours. In contrast, increased SDC-1 expression and correlation with a higher degree of malignancy, as well as expression in connective tissue surrounding the tumour, has also been reported in other tumours. In the present study, we investigated these conflictive findings. A second SDC-1 mRNA isoform was identified and expression of both transcripts was investigated in various normal and malignant tissues.

Based on bioinformatic analysis an additional transcript for SDC-1 mRNA was predicted. The new second transcript has a size of 3.4 kb and differs from the described 2.4 kb mRNA transcript only at the 3′ UTR by an alternative poly-adenylation signal, leaving the ORF unchanged. Hybridisation of either the cDNA probe, which detects both transcripts, or the cDNA probe, which is specific for the newly identified 3.4 kb transcript, to a human MTN blot showed strong mRNA expression in placenta, liver, kidney, and skeletal muscle and weaker signals in colon and lung, indicating that both mRNA transcripts are coexpressed at the same level. No tissue was identified that selectively expresses one or the other transcript. The results confirmed that both SDC-1 transcripts are coexpressed in several human tissues [28].

Next, SDC-1 expression was analysed with the two different cDNA probes on a CPA. In all normal and malignant tissues analysed, both transcripts revealed the same relative expression pattern, confirming the coexpression of both, as previously shown for breast cancer and several other tumour entities [22,28]. SDC-1 was overexpressed in the majority of tumour tissues compared to normal tissues. However, differential gene expression in stromal vs. epithelial cells is not detected when analysing the complete tumour tissue on which the CPA is based, as the tumour cells are often outnumbered by cells of the surrounding stroma, including fat and inflammatory cells. By microdissection, we obtained a >90% separation of carcinoma or normal epithelial cells from other cell types. Surprisingly, Affymetrix oligonucleotide array-based expression profiling from microdissected tumour and normal tissue specimens did not apart from in a few samples, reveal a similarly differential SDC-1 expression pattern between carcinoma and normal epithelial cells. On comparing the results for differential SDC-1 expression in non-enriched vs. enriched tumour and normal tissues, SDC-1 was upregulated in 37.7% of complete tissues compared to only 23.1% of microdissected breast carcinomas. Similarly, for bronchial carcinoma a decrease in cases overexpressing SDC-1, from 28.6% (complete) to 21.4% (microdissected), was observed. The same tendency, from 15.4% (complete) to 7.4% (microdissected), was found in colon carcinoma, and from 25% (complete, but with $P > 0.05$) to 1.9% (microdissected) in prostate carcinoma. This obvious discrepancy in differential expression between non-enriched and microdissected tissue was resolved by ISH. In invasive breast carcinoma, lung carcinoma, and colon carcinoma, SDC-1 expression was detected in stromal cells. This supports the notion that the stromal cells themselves express SDC-1 mRNA, as has been shown recently for invasive breast carcinoma [21] and gastric carcinoma [20]. Therefore, the SDC-1 signal measured on the CPA is most probably to a certain degree derived from stromal cells that either do express more SDC-1 RNA or that indeed outnumber the epithelial cells.

IHC of breast, bladder, colon and lung carcinomas revealed strong staining of the connective stromal cells in a majority of cases. To discover whether cells in the tumour connective tissue are SDC-1 positive, IHC was performed with antibodies specific for marker proteins of epithelial cells (PanCK), myoepithelial cells and myofibroblasts (α-smooth muscle actin) and fibroblasts (vimentin). SDC-1 was coexpressed with vimentin in stromal fibroblasts, whereas the tumour cells themselves were positive for CKs, but negative for vimentin. Additionally, the coexpression of SDC-1 with α-smooth muscle actin as a marker for myofibroblasts and myoepithelial cells led to the conclusion that the stromal cells that express SDC-1 are spindle cells with myofibroblastic differentiation. This indicates a shift of SDC-1 expression from epithelial tumour cells to myofibroblasts within the connective tissue surrounding the tumour cells.

It could be argued that the IHC staining of fibroblasts originates from SDC-1 shed from tumour cells and bound by fibroblasts. However, this is unlikely, since it has been reported that the SDC-1-specific monoclonal antibody B-B4 does not recognise soluble SDC-1 but only the membrane bound form [26], although the epitope of the anti-SDC-1 antibody lies in the extracellular region of the SDC-1 protein and it should theoretically not be able to discriminate between shed and membrane-bound SDC-1. As the same cells that stain positive for SDC-1 protein by IHC are also SDC-1 positive at the mRNA level as demonstrated by ISH, it is most likely that the protein does not originate from SDC-1 shed by other cells but is generated by the stromal cells themselves.

The shift of SDC-1 expression from tumour to stromal cells is in part associated with a decreased expression within epithelial tumour cells. Recently, it has been reported that breast cancer cells can generate their own non-malignant stroma that allows a reciprocal interaction of epithelial tumour cells in order to facilitate tumour cell growth and migration [29]. SDC-1 expression appears to be required to maintain cells in a differentiated epithelial phenotype, as it has been shown that the suppression of endogenous SDC-1 expression in epithelial cells by transfection with antisense cDNA causes a striking change in cell morphology from a flattened

cuboidal shape characteristic of an epithelial cell to an elongated fusiform type. The fusiform transfectants lost expression of E-cadherin, gained the ability to migrate in collagen gels and acquired anchorage-independent growth [30,31].

In conclusion, the decreasing SDC-1 expression and the increasing expression in the spindle cells of the connective tissue surrounding the carcinomas may have two important consequences. One is the change in the phenotype of epithelial tumour cells to mesenchymal characteristics. Since SDC-1 is described as functionally maintaining tumour cells in a differentiated phenotype [18,21], the decrease potentially generates the ability to form a dedifferentiated invasive or migrating tumour cell. The second consequence might be the mobilisation of growth factors from epithelial tumour cell surfaces and extracellular matrix, as shown for hepatocyte growth factor [32] and basic fibroblast growth factor [17]. The shift of SDC-1 expression to the spindle cells with mesenchymal differentiation could originate the recruitment of soluble factors needed for the establishment of the cancer-associated non-malignant stroma [29]. Finally, the cancer-associated non-malignant stroma may contribute to tumour cell invasion and the development of metastases. However, stromal SDC-1 expression is observed in several but not all tumour types. Therefore, the biological effects of SDC-1 might critically depend on the cell type in which SDC-1 is expressed.

In summary, we isolated an alternative mRNA transcript coding for a SDC-1 protein with the same ORF. We analysed the expression pattern of SDC-1 in microdissected and complete tumour and normal tissue samples of different cancers and demonstrated a shift of SDC-1 expression from tumour cells to stromal myofibroblasts within breast carcinoma, colon carcinoma, bladder carcinoma and lung carcinoma. As knowledge about tumour–stroma interactions is becoming more and more important, it is relevant to consider a future analysis of the differential roles of tumour stroma and stroma surrounding normal epithelial cells.

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