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ORIGINAL ARTICLE

Coexpression and nuclear colocalization of metastasis-promoting protein S100A4 and p53 without mutual regulation in colorectal carcinoma

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Abstract Nuclear localization of the metastasis-associated protein S100A4 has been shown to correlate with advanced disease stage in primary colorectal carcinomas (CRC), but nuclear function and its relevance for the metastatic capacity of tumor cells is still unclear. Among several nuclear interacting protein partners suggested for S100A4, the tumor suppressor protein p53 has attracted particular interest, and previous studies suggest direct and indirect modes of interaction between the two proteins. The present study was undertaken to assess coexpression and potential interaction in CRC. *TP53* mutational status and S100A4 expression were investigated in a selected series of primary CRC specimens ($n = 40$) and cell lines ($n = 17$) using DNA sequencing, western blot, and double immunostaining. Additionally, S100A4 and p53 were experimentally up- and down-regulated in vitro to assess reciprocal effects. For the first time, S100A4 and p53 coexpression was demonstrated in individual CRC cells, with nuclear colocalization as a particularly interesting feature. In contrast to previous studies, no correlation was observed between *TP53* mutational status and S100A4 expression, and no evidence was obtained to support reciprocal regulation between the two molecules in the HCT116 isogenic cell line model. In conclusion, S100A4 and p53 were shown to be colocalized in individual nuclei of CRC cells, and it might be speculated whether the proteins interact in this subcellular compartment.

Keywords Colorectal neoplasms · S100A4 · p53 · *TP53* · Metastasis

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Introduction

The S100A4 protein belongs to the S100 protein family, which in humans comprises at least 20 small, acidic Ca^{2+}-binding proteins, exhibiting distinct expression patterns, tissue distribution and biological properties (Donato 2003; Marenholz et al. 2004). The S100A4 gene product has been convincingly linked to the invasive and metastatic phenotype of cancer cells through experimental data derived from transfections with expression constructs (Davies et al. 1993; Ford et al. 1995; Maelandsmo et al. 1996; Takenaga et al. 1997) and studies of transgenic animals (Ambartsumian et al. 1996; Davies et al. 1996). In addition, clinical evidence has indicated a correlation between augmented S100A4 expression and poor prognosis in several cancer types, including colorectal carcinoma (CRC) (Andersen et al. 2004; Gongoll et al. 2002; Rudland et al. 2000). The S100A4 protein has been shown to influence various

biological functions with potential implications for the metastatic phenotype, such as invasion, cell motility and angiogenesis (Garrett et al. 2006; Helfman et al. 2005; Sherbet and Lakshmi 1998). However, the exact functions of S100A4 and how it exerts its metastasis-promoting effects remain incompletely understood.

The tumor suppressor protein p53 plays a pivotal role in the maintenance and regulation of normal cellular functions, and mutations in *TP53* are observed in approximately 50% of human CRC (Iacopetta et al. 2006). Through steps of stabilization, phosphorylation, and nuclear translocation, p53 is activated in response to various cellular stress signals, forming a tetrameric transcription factor able to modulate the expression of numerous downstream genes. A large set of target genes have been identified, and the corresponding proteins are involved in diverse functions, such as cell cycle regulation, apoptosis, cellular signaling and regulation of extracellular matrix [reviewed in Aylon and Oren (2007), Levine (1997)]. Additionally, to mediate its pleiotropic effects, p53 interacts with a number of proteins, and has also recently been implicated in regulation of angiogenic transcription factors and vascular development, which are important features of metastasis (Teodoro et al. 2007).

Certain key observations have instigated research towards a possible physical and regulatory interaction between S100A4 and p53. When S100A4 was transfected into *TP53* wt cell lines, apoptotic cell death was observed, whereas stable clones were constructed successfully in *TP53* mutated cell lines (Grigorian et al. 2001). It was hypothesized that coexistence of S100A4 and wt *TP53* would be lethal to tumor cells, and that the implied selection pressure towards an aggressive, *TP53* mutated and metastatic phenotype, in part, could explain the metastasis-promoting effects of S100A4. Additionally, interactions between p53 and two other S100 family members, S100B and S100A2, have been demonstrated, and both these S100 proteins may regulate p53 at the transcriptional level and by way of protein–protein interactions (Fernandez-Fernandez et al. 2005; Lin et al. 2001, 2004; Mueller et al. 2005). Since neither of these proteins are expressed in colorectal epithelium or its malignant derivatives (Bronckart et al. 2001), one might speculate that S100A4 could be responsible for the execution of "S100-functions" with respect to interaction with p53 in CRC. Substantial research has documented in vitro physical interactions between S100A4 and p53, and S100A4 has been shown to influence *TP53* transcriptional regulation in experimental settings (Chen et al. 2001; Fernandez-Fernandez et al. 2005; Grigorian et al. 2001). Finally, our previous finding that nuclear localization of S100A4 correlated with tumor stage in CRC led us to consider whether a potential S100A4-p53 interaction might be a nuclear phenomenon (Flatmark et al. 2003). The objective of the present study was to examine the correlation between S100A4 expression and *TP53* mutational status in primary CRC and cell lines, and assess potential reciprocal influence between the two proteins in experimental model systems.

Materials and methods

CRC samples

From a clinically representative series of primary CRC, 40 cases were selected, including ten tumors from each Duke's stage (Table 2). Within each group, five tumors were negative and five were positive for nuclear S100A4, according to previous immunohistochemical analysis (Flatmark et al. 2003). All tumors with nuclear S100A4 and nine of the nucleus negative samples also expressed cytoplasmic S100A4. Tumor tissue was collected at the time of surgery, snap frozen in liquid nitrogen and stored at −80°C until use. For each sample, the proportion of tumor cells was assessed in frozen sections neighboring the tissue used for DNA extraction. If possible, microscopic-guided rough dissection was performed to remove normal tissue prior to DNA extraction. The study was approved by the Regional Committee for Medical Research Ethics Regional Ethics Committee (Approval#S-98080) and informed consent was obtained.

Cell lines

Co205, HCT15, Colo320DM, SW480, SW620, $CaCo_2$, HT29, RKO, SW48 and WiDr cell lines were purchased from ATCC (Manassas, VA, USA). HCT116 cells were purchased from ATCC; but when specified, two isogenic variants obtained from Dr. Bert Vogelstein (The Sidney Kimmel Comprehensive Cancer Centre, The Johns Hopkins Medical Institutions, Baltimore, MD, USA) were used: HCT116 *TP53* knock-out (*TP53*−/−) and HCT116 wild-type (*TP53*+/+) (Bunz et al. 1999). KM20L2 and HCC2998 were kindly provided by Michael R. Boyd (National Cancer Institute, Frederick, MD, USA). Co115 cells were obtained from B. Sordat (Epalinges, Lausanne, Switzerland), whereas EB, LS174T and TC7 cell lines were acquired from Richard Hamelin (INSERM, Paris, France). Cells were cultivated in RPMI 1640 (BioWhittaker, Lonza Verviers, Belgium) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 20 mM Hepes (BioWittaker) and 2 mM Glutamax (Gibco, Invitrogen, Norway). All cell lines were negative for mycoplasma infection. *TP53* mutational status of cell lines is available at the *TP53* web site (http://p53.free.fr/index.html), except

for cell lines KM20L2 and Co115 which were found to harbor mutated (k273; CGT-CAT; Arg-His) and wt *TP53*, respectively (for methods see below).

TP53 mutation analysis

TP53 mutation status within the evolutionary conserved DNA binding domain, encoded by exons 5–8, was assessed for each tumor sample. PCR products with aberrant migrating bands detected by temporal temperature gradient gel electrophoresis (TTGE) (Sorlie et al. 2005) were indicative of a mutation. New PCR products from the same DNA stock solution were then submitted to direct sequencing of the targeted exon using dideoxy sequencing (ABI PRISM TM373 DNA Sequencer, Applied Biosystems) to identify the specific sequence change (mutation or polymorphism). The four exons were amplified by PCR using the standard protocol for HotStar DNA Polymerase (Qiagen, GmbH, Hilden, Germany) followed by sequencing with primers and annealing temperatures as shown in Supplementary table 1. In one sample, an aberrant band was detected on TTGE, but the mutation could not be confirmed by sequencing, and was thus interpreted as wt. No wt conclusions were drawn from analysis of samples containing less than 20% tumor cells.

Double immunohistochemistry (DIHC) and double immunofluorescence (DIF)

Five micrometer formalin-fixed, paraffin-embedded sections were deparaffinized, rehydrated and antigen retrieved in Tris–EDTA buffer, pH 9.0 in Pascal pressure chamber (DAKO) at 120–125°C, 18 psi (30 s) for cell pellets, and by microwaving at 750 W (9 min) and 250 W (15 min) for tumor tissue. For DIHC, sections were blocked with 10% goat serum in TBST (Sigma, St. Louis, Missouri) (30 min) and afterwards with Dual Endogenous Enzyme Block (DAKO) (10 min) prior to the addition of primary antibody #1. After washing, secondary antibody Polymer/HRP (EnVision G|2 Double System Rabbit/Mouse DAB+/Permanent red kit, DAKO) was added, followed by visualization with 3,3′-diaminobenzidine (10 min). Doublestain block (DAKO) was added (3 min) before primary antibody #2. To enhance the second primary antibody signal, Link reagent (EnVision G|2 Double System Kit) was added (30 min) prior to secondary antibody Polymer/AP (EnVision G|2 Double System Kit), and visualization was performed with liquid permanent red (10 µl/ml) (DAKO) diluted with Levamisole (30 µl/ml) (DAKO) in substrate buffer (8 min).

For DIF, after 30 min blocking with 10% goat serum, primary antibodies were applied in mixture, followed by washing and addition of fluorochrome conjugated secondary antibodies in mixture. Slides were mounted in DAPI-Vectashield mounting medium (Vector Laboratories Ltd, Peterborough, UK) and analyzed by confocal laser scanning microscopy (Axiovert25 inverted microscope, Carl Zeiss MicroImaging, Göttingen, Germany). Specimens incubated with Antibody Diluent alone, or with single primary antibody were used as negative controls (for conditions see Table 1).

Table 1 Antibodies and conditions used for western blot (WB), double immunohistochemistry (DIHC) and double immunofluorescence (DIF)

Application	Target	M/P (species)	Catalog/Clone	Source	Dilution	Buffer	Conditions
WB	S100A4	P (rabbit)	A5114	DAKO	1/300	TBST	1 h, RT
WB	S100A4	M	22.3	In-house[a]	1/300	TBST	1 h, RT
WB	p53	M	SC-6243	Santa Cruz	1/3000	TBST	1 h, RT
WB	α-tubulin	M	CP06	Calbiochem	1/300	TBST	1 h, RT
DIHC	S100A4	M	20.1	In-house[a]	1/200	AD	1 h, RT
DIHC	p53	M	DO-7	DAKO	1/1000	AD	30 min, RT
DIF	S100A4	P (rabbit)	A5114	DAKO	1/50	TBS	1 h, RT
DIF	p53	M	DO-7	DAKO	1/10	TBS	1 h, RT
DIF	Anti-mouse	P (goat)	Alexa Fluor 594	Invitrogen	1/200	AD	30 min, RT
DIF	Anti-rabbit	P (goat)	Alexa Fluor 488	Invitrogen	1/200	AD	30 min, RT

DAKO, Glostrup, Denmark; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Calbiochem, Merck Biosciences GmbH, Germany; Invitrogen, Carlsbad, CA, USA

M monoclonal; *P* polyclonal; *TBST* tris buffered saline with 0.25% Tween 20 and 5% non-fat dry milk; *TBS* tris-buffered saline; *AD* antibody diluent, DAKO; *RT* room temperature

[a] Flatmark et al. 2004

Transfection experiments

Transfections constructs for wt and mutated *TP53* were a gift from Dr. Bert Vogelstein (The Sidney Kimmel Comprehensive Cancer Centre, The Johns Hopkins Medical Institutions, Baltimore, MD, USA) (Baker et al. 1990). The S100A4 expression construct was made by PCR amplifying the insert from S100A4/pBlusescript (kindly provided by Dr. Heizmann, Department of Pediatrics, University of Zürich, Zurich, Switzerland), and subsequently ligating the product into pcDNA3.1 (Invitrogen). Transient transfections of S100A4 and *TP53* wt and mutated constructs in HCT116 were performed by electroporation, using 4 mm cuvette gap, 240 V, 26 ms, 1 pulse exposure (BTX, ECM 830, San Diego, CA, USA). For transfection of 2×10^6 cells in RPMI/FBS, 2.5–10 μg pcDNA3.1/S100A4, the corresponding pcDNA3.1 control vector, pcDNA3.1/wt p53 or pcDNA3.1/mutated p53, were used. Following electroporation, 1.5×10^6 cells were seeded in serum-containing medium in T25 flasks.

Silencing of endogenous expression of S100A4 was performed using siRNA duplexes; si-S100A4: 5-UGA-GCA-AGU-UCA-AUA-AAG-A-3 (against position 481–499, in the 3′UTR area), and si-scrambled control: 5-CGC-AUA-AGU-GAA-AUA-GAA-U-3 (Eurogentec, Belgium). Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 5×10^5 cells were seeded in T25 flasks and grown overnight before adding 100 nM siRNA/5 μl Lipofectamine in RPMI/w/o FBS. After 6 h medium containing serum was added.

Cellular responses to ultraviolet (UV) radiation

48 or 72 h after S100A4 or si-RNA transfection, respectively, HCT116 cells were exposed to a fixed dose of UV irradiation while in PBS (12 J/m^2, 254 nm) in a GS gene linker instrument (Bio-Rad, Hercules, CA, USA) and left for 6 h in complete medium. Total cell lysates were harvested, or cell pellets were formalin fixed and paraffin embedded for DIF or DIHC studies. For all experiments, mock-irradiated control cultures were handled identically. Cell pellets were sectioned and immunostained as described above, and staining of tumor cells was quantitatively evaluated by counting six fields for each sample at 20× magnification (median number of cells per field was 128, range 87–235) (Table 3).

Cell lysis and western blot analysis

Total cell lysates were made by adding lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 0.1% NP-40, 10 μg/ml Leupeptide Hemisulfate, Aprotinin, Pepstatin A, 20 mM β-glycerolphosphate, 1 mM PMSF, 1 mM sodium orthovanadate and 100 mM sodium fluoride) to dry cell pellets, and following sonication and centrifugation, supernatant was stored at −80°C. Lysates were separated by SDS-PAGE (4–12% NuPAGE, Invitrogen), transferred to 0.45 μM PVDF-membranes (Millipore, MA, USA) and probed with antibodies against S100A4, p53 and α-tubulin (as internal control) (for conditions see Table 1). Immune complexes were detected with Super Signal chemiluminescence detection system (Pierce, Rockford, IL, USA). Scanning and densitometry analysis was performed using a GS-800 Calibrated Densitometer and Quanti-One 4.6.6 software (Bio-Rad).

Statistics

All group comparisons were performed using the Mann–Whitney *U* test (SPSS version 15.0; SPSS, Chicago, Ill. USA), and $P \leq 0.05$ was considered statistically significant.

Results

TP53 mutational status and S100A4 expression in CRC

Approximately half (19 of 40) of the CRC samples exhibited *TP53* mutations (Table 2), in agreement with previous results (Borresen-Dale et al. 1998; Iacopetta et al. 2006). The 19 mutations are listed in Supplementary table 2. Mutations were present in 43, 58 and 43% in tumors from the rectum, left colon, and right colon, respectively, in accordance with previous studies from us and others (Borresen-Dale et al. 1998). In tumors that expressed nuclear S100A4, mutated *TP53* was present in 45% of the cases (9 of 20), whereas wild-type was found in 55% (11 of 20). Similarly, no difference in *TP53* mutational frequency could be detected between tumors with cytoplasmic S100A4.

S100A4 expression in cell lines with wt and mutated *TP53*

To assess whether S100A4 expression was dependent on p53 mutational status in human CRC cell lines, protein lysates from 7 wt and 10 mutated cell lines were analyzed by Western blot. Variable amounts of S100A4 were detected in most of the cell lines as shown in Fig. 1. Although the four cell lines with the highest S100A4 levels were in the *TP53* mutated group (KM20L2, SW620, SW480 and Co205), six of the seven wt lines had detectable S100A4 with expression levels similar to the remaining mutated cell lines. Only two cell lines, RKO and

Table 2 *TP53* mutational status according to tumor stage, localization and nuclear and cytoplasmic expression of S100A4

	TP53 mutational status	
	Mutated number (%)	Wild-type number (%)
Dukes' stage		
A	6 (60)	4 (40)
B	5 (50)	5 (50)
C	6 (60)	4 (40)
D	2 (20)	8 (80)
Localization		
Rectum	6 (43)	8 (57)
Left colon	7 (58)	5 (42)
Right colon	6 (43)	8 (57)
S100A4 expression		
N S100A4+	9 (45)	11 (55)
N S100A4−	10 (50)	10 (50)
C S100A4+	15 (52)	14 (48)
C S100A4−	4 (36)	7 (64)
All samples	19 (48)	21 (52)

N nuclear, *C* cytoplasmic

HCT15, were entirely negative for S100A4, harboring wt and mutated *TP53*, respectively.

Coexpression of S100A4 and p53 in HCT116 cells and patient samples

The presence of S100A4 in cell lines with wt *TP53* was demonstrated by western blot, but to assess whether p53 and S100A4 were present in the same cells, cellular imaging strategies were applied (Fig. 2). Co-immunostaining performed in HCT116 cells with or without induction of p53 by UV irradiation revealed p53 expression exclusively in the nuclei of HCT116 cells, whereas S100A4 exhibited nuclear and cytoplasmic expression. A sixfold increase in the fraction of cells expressing p53 was observed upon UV exposure, while S100A4 expression was essentially unchanged (Table 3). In unirradiated cells, 1.5% of cells exhibited expression of both p53 and S100A4, and the fraction of double-stained cells increased to 4.6% upon UV exposure. The pattern of coexpression varied, as co-immunostaining in nuclei was observed in some cells, while concomitant presence of nuclear p53 and cytoplasmic S100A4 was detected in other cells. Two patient samples were similarly analyzed by DIHC, showing variable nuclear expression of wt p53, and occasional cells (<1%) were observed with simultaneous expression of mainly nuclear S100A4.

Experimental regulation of S100A4 and p53

To assess potential reciprocal regulation between S100A4 and p53 in vitro, isogenic variants of the CRC cell line HCT116 harboring wt (*TP53*+/+) and knock-out p53 (*TP53*−/−) were used. Protein levels of S100A4 or p53 were experimentally altered using transient transfection or by exposing cells to genotoxic stress by UV radiation. In accordance with previous results (Daoud et al. 2003), high and very low basal levels of S100A4 were detected in wt (*TP53*+/+) and knock-out (*TP53*−/−) cells, respectively (Fig. 3a).

Restoring p53 expression in HCT116 *TP53* knock-out cells by transfection with an expression construct encoding wt p53 did not alter S100A4 levels (Fig. 3b). Furthermore, by performing the opposite experiment, transfecting S100A4 into the *TP53* wt cells, an anticipated up-regulation of S100A4, but no changes in p53 protein levels were observed (Fig. 4; Supplementary Fig. 1). Similarly, siRNA-mediated down regulation of S100A4 did not affect constitutive p53 expression in HCT116 wt cells (Fig. 5; Supplementary Fig. 2).

Even if augmenting p53 expression by transfection did not affect S100A4 expression levels, manipulation of endogenous p53 might in theory induce biological effects that could not be achieved with exogenously introduced protein. Thus, UV irradiation of *TP53* wt cells was performed, resulting in increased amounts of p53, while S100A4 expression remained unchanged (Fig. 4). To further assess whether manipulating S100A4 levels could influence the cells' ability to activate p53 following genotoxic stress, S100A4 was experimentally up- or down-regulated in HCT116 *TP53* wt cells by transfection strategies prior to UV exposure. UV dependent increase of p53 expression was not influenced by altered S100A4

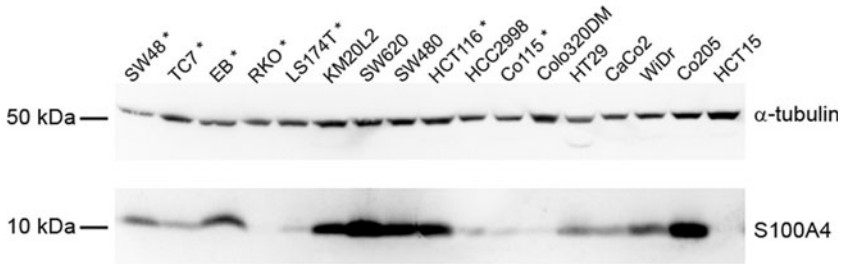
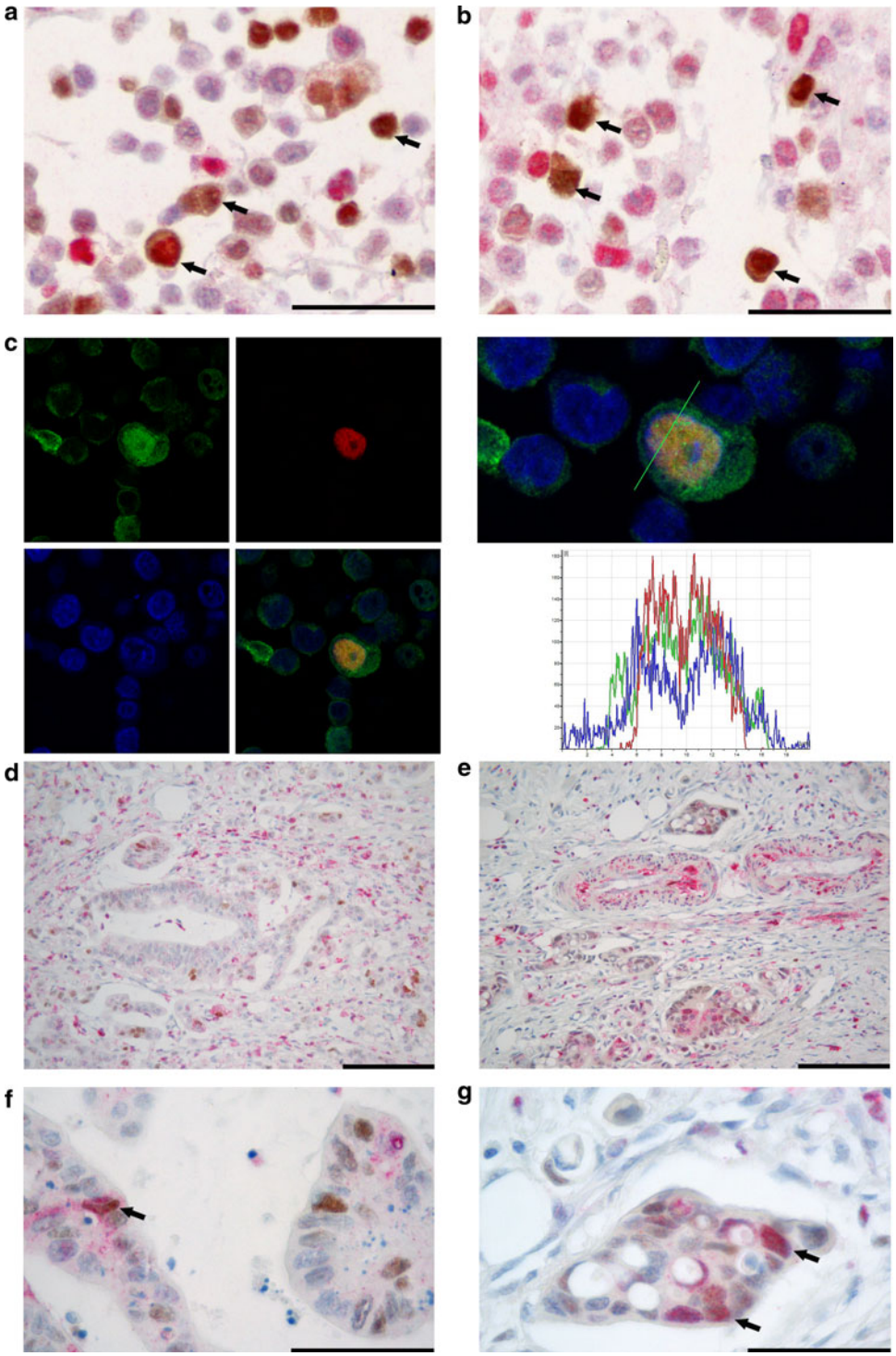


Fig. 1 Western blot of SDS PAGE separated protein lysates from seven *TP53* wt (*asterisk*) and ten mutated human colorectal carcinoma cell lines, immunostained with a mAb against S100A4. Staining for α-tubulin was used as internal standard



expression after transfection with the S100A4 expression construct (Fig. 4) or with siRNA against S100A4 (Fig. 5).

Discussion

In the examined CRC samples, no association was observed between nuclear or cytoplasmic S100A4 expression and *TP53* mutational status. Moreover, six of the seven *TP53* wt cell lines expressed S100A4, as assessed by immunoblotting of total protein lysates. The idea that S100A4 expression could induce cell death in *TP53* wt cells, thus contributing to selection of a metastatic phenotype harboring mutated *TP53* and S100A4 is appealing, but the hypothesis has not been tested in clinical samples or on the single cell level. According to this theory, one would expect

◀ **Fig. 2** Double immunohistochemistry (DIHC) and double immunofluorescence (DIF) images demonstrating concomitant presence of S100A4 and wt p53 in HCT116 cells. **a** Untreated HCT116 cells were formalin fixed and paraffin embedded and sections were probed with antibodies against S100A4 and p53, visualizing nuclear accumulation of p53 (*red*) and both cytoplasmic and nuclear expression of S100A4 (*brown*) (63×, *scale bars* 50 μm). In some cells (*arrows*) double staining was observed, with nuclear p53 and nuclear S100A4 and/or cytoplasmic S100A4. **b** When HCT116 cells were exposed to UV irradiation, nuclear accumulation of p53 (*red*) was more frequently observed (63×, *scale bars* 50 μm), and relative to the increase in p53 expression, the number of double stained cells also increased (see Table 3 for quantification). c Confocal images of untreated HCT116 cells demonstrating colocalization of S100A4 (*green*) and p53 (*red*) in a representative cell nucleus (merged picture is *bottom right* of the quadruplet) and simultaneous expression of cytoplasmic S100A4 in the same cell as well as a number of neighboring cells. DAPI-stained nuclei appear *blue*. (63× and Z-depth was 300 nm). Colocalization was also verified by quantification of fluorescence in the indicated transverse section as depicted in the histogram. **d**–**g** DIHC images from two colorectal tumors that expressed S100A4 in the original immunohistochemical study (Flatmark et al. 2003) and harbored wt *TP53* as assessed by mutational analysis. **d**, **e** Low magnification (20×, *scale bars* 100 μm) images giving an overview of tumors from patient number 207 and 186, respectively. **f**, **g** High magnification (63×, *scale bars* 50 μm) details from each tumor. Nuclear expression of p53 (*brown*) was present in both tumors, but was more intense in the sample from patient 186 than in the one from patient 207. Nuclear and cytoplasmic S100A4 (*red*) was present in both tumors, and concomitant expression, mainly nuclear, of S100A4 and p53 was visualized in occasional tumor cells (*arrows*). To visualize the relatively weak p53 staining in the two patient specimens, the order of primary antibody application was switched compared with the cell pellet analysis in **a** and **b**

the favored combinations in tumor samples to be wt *TP53*/S100A4 negative and mutated *TP53*/S100A4 positive. In the present study, expression of nuclear and cytoplasmic S100A4 was equally frequent in *TP53* wt and mutated samples, and thus the results did not corroborate the hypothesis that the presence of S100A4 and wt *TP53* is mutually exclusive.

Because of tumor heterogeneity, variable abundance of normal cells in clinical samples and the possibility of mutations existing outside the examined region of the p53 gene, there could be a risk that existing mutations might not be detected. Conversely, since some mutations are associated with production of functional p53 and the exact effect of every mutation has not been clarified, detected mutations might not necessarily indicate complete loss of p53 function (Iacopetta et al. 2006). The TTGE screening method has a limit of detection (LOD) of ~5% tumor cells present in the sample (Sorlie et al. 2005), while for the direct sequencing method, LOD is ~20% (unpublished results; Dahl et al. 2006). Since no wt conclusion was drawn from samples containing less that 20% tumor cells, the chance of missing mutations in the examined region is considered negligible. Moreover, more than 80% of all mutations in *TP53* are reported to be situated within exons 5 through 8, involving most of the DNA binding domain which is crucial for the transactivating ability of p53 (Walker et al. 1999). Therefore, the chance of false wt conclusions in tumor samples is low and the risk of missing mutations relevant for p53 function should be even lower, while some of the mutated samples might potentially retain p53 function.

Using co-immunostaining strategies with antibodies against S100A4 and p53, we were able, for the first time, to demonstrate simultaneous presence of the two proteins in individual CRC cells, and in particular, nuclear coexpression was noted (Fig. 2). We previously showed that nuclear expression of S100A4 was correlated to advanced disease stage in CRC (Flatmark et al. 2003), and preliminary results indicate that nuclear S100A4 is an independent predictor of adverse prognosis in the same patient cohort (Boye, manuscript in preparation). Thus, the possibility of molecular interaction between S100A4 and other nuclear proteins, such as p53, is intriguing. While recently disputed, a substantial amount of data from in vitro experiments using different methods and cell systems supports a direct physical interaction between p53 and S100A4, involving binding of S100A4 to the C terminus of p53, with potential implications for cellular localization, DNA affinity, p53 phosphorylation by PKC as well as downstream target activation (Chen et al. 2001; Fernandez-Fernandez et al. 2005; Grigorian et al. 2001; van Dieck et al. 2009). The only in vivo finding suggesting direct protein–protein interaction involved co-immunoprecipitation from cells harboring mutant *TP53* (Grigorian et al. 2001), while co-immunoprecipitation of S100A4 with wt p53 has so far not been demonstrated. This could be attributed to instability of wt p53 and technical issues, and

Table 3 Immunohistochemical analysis of p53 and S100A4 staining in HCT116 cells

	Mean percentage positively stained HCT116 cells (SD)			
	p53	N S100A4	C S100A4	p53 and S100A4
Untreated cells	4.7 (1.0)	6.6 (2.5)	11.0 (3.9)	1.5 (0.9)
UV treated cells	31.7 (6.9)	7.5 (4.1)	13.9 (4.7)	4.6 (3.0)

Tumor cells were counted at 20× magnification, six fields for each sample (median number of cells per field was 128, range 87–235)

N nuclear, *C* cytoplasmic, *SD* standard deviation

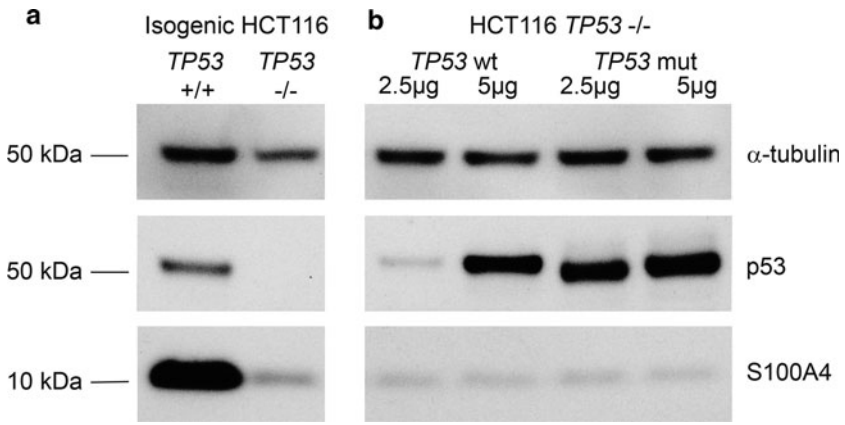


Fig. 3 Western blot showing the expression of S100A4 and p53 in the HCT116 isogenic cell model and the effects of transfecting wt or mutated *TP53* into HCT116 *TP53*−/− cells. **a** The *TP53* wt cells from the isogenic HCT116 cell model expressed high levels of S100A4 and detectable p53, while the *TP53* knock-out cells had very low amounts of S100A4 and no p53 protein was detected. **b** Reconstituting p53 expression in the knock-out cells by transfection with wt or mutated construct did not influence S100A4 expression levels

interaction between the two proteins could still take place and be of biological relevance, for instance, in the cell nucleus.

An alternative and more indirect possibility for molecular interaction between S100A4 and p53 could be through transcriptional regulation. However, results from in vitro experiments indicated that although S100A4 and p53 were indeed present in the same cells and even in the same nuclei, no reciprocal regulation was observed. These findings are in contrast to results from studies of other S100 proteins, such as S100B and S100A2. Increased S100B levels in malignant melanoma cell lines dose dependently down-regulated p53 and inhibited p53-mediated cellular effects, whereas p53 levels and function were restored when S100B was down-regulated by antisense RNA. Additionally, over-expression of S100B prevented induction of p53, p21 and MDM2 in response to bleomycin-induced DNA damage. S100B transcription was up-regulated by p53 through interaction with consensus p53 binding sites in the S100B promoter, and also, UV induction of p53 led to increased mRNA and protein levels of S100B (Lin et al. 2001, 2004). The evidence supporting mutual regulation of S100A2 and p53 is less substantial, but in osteosarcoma cells, up-regulation of S100A2 on the transcriptional level was described through p53 binding sites in the promoter sequence (Tan et al. 1999). Since the S100A4 promoter also contains putative p53 binding sites (Parker et al. 1994), up-regulation of p53 by transfection and UV irradiation could be expected to influence S100A4 transcription, but no such effects were observed. Hence, although comparable experimental conditions were applied, we did not observe any of the profound effects that would be expected if S100A4 had a regulatory interaction with p53 comparable to that of S100B or S100A2. However, although the isogenic HCT116 cell

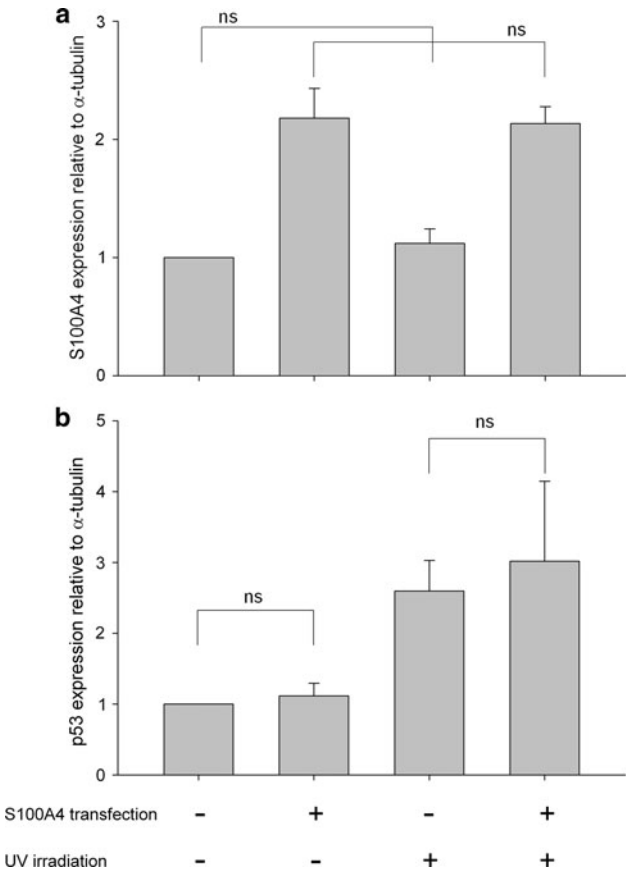


Fig. 4 The figure shows mean values (+SEM) for S100A4 and p53 expression relative to respective α-tubulin staining derived from densitometry scans of western blots from three independent experiments (*ns* not significant). **a** Transfection with S100A4 resulted in significant ($P \leq 0.05$) up-regulation of S100A4 expression compared to mock transfected cells, while UV irradiation did not influence S100A4 expression. **b** UV exposure gave rise to p53 induction ($P \leq 0.05$) regardless of S100A4 protein expression levels, while S100A4 transfection did not influence constitutive or UV induced amounts of p53

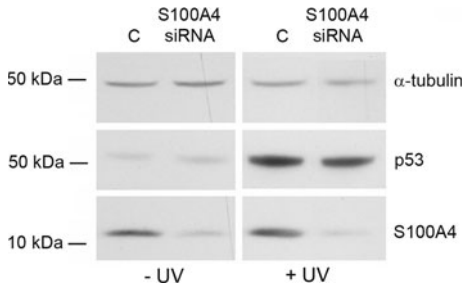


Fig. 5 Representative western blots showing expression of S100A4 and p53 in HCT116 cells upon transfection with siRNA against S100A4 or scrambled siRNA (*C*) with or without subsequent UV exposure. Experimental down-regulation of S100A4 with siRNA did not influence baseline p53 expression levels or the p53 induction achieved by UV irradiation

model is an established and much-used tool for the study of p53-specific interactions, results could be limited to the experimental system and do not exclude interaction between p53 and S100A4.

Since nuclear localization of the metastasis-related protein S100A4 is clinically relevant in CRC, identification of putative nuclear interacting protein partners is of interest. In the present study, nuclear and cytoplasmic S100A4 were expressed equally frequently in *TP53* wt and mutated tumors and cell lines, and coexpression of the two proteins was demonstrated in CRC cells and in clinical specimens. Although experimental manipulation of S100A4 and p53 expression did not show evidence for mutual regulation in the isogenic HCT116 cell lines, a role for direct or indirect interaction between S100A4 and p53 cannot be excluded. Simultaneous detection of S100A4 and p53 in the nucleus of CRC cells lends support to our initial theory that interaction between p53 and S100A4 could take place in the cell nucleus, but potential biological implications remain obscure.

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