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Diagnostic and Prognostic Value of Metastasis Inducer S100A4 Transcripts in Plasma of Colon, Rectal, and Gastric Cancer Patients

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Early detection of tumors and metastases is critical for improving treatment strategies and patient outcomes. The development of molecular markers and simple tests that are clinically applicable for detection, prognostication, and therapy monitoring is strongly needed. The gene S100A4 has long been known to act as a metastasis inducer. High S100A4 levels in the primary tumor are prognostic for metachronous metastasis and correlate with reduced patient survival. We provide, for the first time, a plasma-based assay for transcript quantification of S100A4 in gastrointestinal patients' plasma. We conducted a study to define the diagnostic and prognostic power of S100A4 transcripts using 466 plasma samples from colon, rectal, and gastric cancer patients. Plasma was separated, RNA was isolated, and S100A4 mRNA was determined by quantitative RT-PCR. S100A4 transcripts were increased in cancer patients of each entity ($P < 0.0001$) and all disease stages ($P < 0.05$), compared with tumor-free volunteers (sensitivities of 96%, 74%, and 90% and specificities of 59%, 82%, and 71%, for colon, rectal, and gastric cancer patients, respectively). Prospectively analyzed follow-up patients who later experienced metastasis showed higher S100A4 levels than follow-up patients without metastasis. Disease-free survival was decreased in high S100A4-expressing follow-up colorectal cancer patients ($P = 0.013$). In summary, we developed a method for quantitative S100A4 transcript determination in plasma that allows clinical application routinely. We demonstrated the diagnostic and prognostic potential of this method for early defining cancer staging and patients' risk for metastasis. *(J Mol Diagn 2011, 13:189–198; DOI: 10.1016/j.jmoldx.2010.10.002)*

Gastrointestinal cancers, such as colon, rectal, and gastric cancers, belong to the malignancies with the highest incidences and mortalities worldwide. Approximately 90% of all cancer deaths arise from the metastatic dissemination of primary tumors.[1,2] To date, primary colorectal carcinomas cannot be sufficiently distinguished with respect to clinical outcome parameters, such as local recurrence and metastasis, by means of conventional clinical and histopathological/immunhistochemical examination. The tumor-node-metastasis (TNM) classification represents the main tool for identifying prognostic differences among patients with early-stage colorectal cancer.[3] This is also true for gastric cancer, currently evaluated by histological staging as well.[4]

Therefore, early detection of tumors and metastasis is critical for improving treatment strategies and patient outcomes. There is a clear need for development of molecular markers and of simple tests that can clinically be used for detection, prognostication, and therapy monitoring of cancer. A noninvasive blood test for the early identification of high-risk cancer patients is therefore of special interest. Circulating nucleic acids and, in particular, cell-free mRNA can be detected in plasma and permit plasma-based expression profiling.[5–9] It was recently reported that extracellular plasma RNA from colon cancer patients is confined in a vesicle-like structure and is mRNA-enriched.[10] The quantitative detection of tumor-derived transcripts in blood might allow the identification of occult tumors and metastases in apparently healthy individuals. Moreover, blood-based diagnostic methods are not only useful for snapshots, such as tumor marker determination in patients' biopsy samples, but allow monitoring of disease progression, therapy efficacy, and response. Cancer diagnostics with plasma-based expression profiling is quick, clinically applicable, and cost effective, and is particularly attractive from the point of view of patient comfort. However, no reli-

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Table 1. Colon Cancer Patient Characteristics and S100A4 Transcript Levels

		Colon cancer patients					
		Day of diagnosis			During follow-up		
	Tumor-free volunteers	Newly diagnosed primary tumor without metastasis	Newly diagnosed primary tumor with synchronous metastasis	Newly diagnosed metachronous metastasis	Follow-up without metastasis	Follow-up with metastasis	All
Blood samples, *n*	51	13	10	5	145	12	185
UICC I, %		47	0	0	25	0	23
UICC II, %		38	0	60	45	69	44
UICC III, %		15	0	40	28	31	26
UICC IV, %		0	100	0	2	0	7
Adjuvant therapy		2/13	—	1/5	48/145	5/12	56/185
Follow-up, median, days		726	181	274	742	695	729
Age, median (range), years	60 (27–86)	64 (51–76)	66 (37–78)	64 (49–76)	68 (37–83)	65 (54–67)	67 (37–83)
Sex, male/female	37/14	10/3	6/4	1/4	86/59	9/3	112/73
S100A4 mRNA expression, % calibrator [median]	0.219	0.347	0.6825	0.631	0.403	0.67	0.434
P, vs tumor-free volunteers		0.0055	<0.0001	0.0023	<0.0001	<0.0001	<0.0001

able noninvasive blood-based methods are routinely used for the detection and grading of cancer.

S100A4 (metastasin, mts1) plays an important role as metastasis inducer.[11,12] It is a member of the multigene S100 family of calcium-binding proteins. Intracellular S100A4 interacts with proteins of the cytoskeleton, such as actin filaments, nonmuscle tropomyosin, and nonmuscle myosin II, thereby directly increasing cell motility.[13–15] S100A4 also induces a migratory phenotype by affecting cell adhesion via binding to liprin β1.[16] Moreover, S100A4 binds to the tumor suppressor p53 and modulates its transcriptional activity.[17] Extracellular S100A4 activates expression of several matrix metalloproteinases, thereby enabling cell invasion into adjacent tissues and facilitating angiogenesis.[18,19]

High levels of S100A4 correlate with reduced patient survival and poor prognosis in several types of cancer.[20–23] In colorectal cancer, high S100A4 expressions correlate with aggressive tumor growth and poor prognosis.[24–30] Overexpression of S100A4 is also related to aggressiveness and metastasis in gastric cancer.[31–34]

We demonstrated previously that S100A4 transcripts, when quantitatively determined in the nonmetastasized primary tumor, have potential value for prognosis of metastasis formation in colon cancer patients.[29] Here we provide, for the first time, a reliable and simple plasma-based assay for transcript quantification of the metastasis-promoting gene S100A4. We demonstrate its applicability and diagnostic value for newly diagnosed or already treated patients with colorectal and gastric cancer. Moreover, the prognostic value of S100A4 transcript for metastasis formation and disease-free survival (DFS) for follow-up patients is shown.

Materials and Methods

Patient Data and Classification

Patients with colon, rectal, or gastric cancer who were treated at the Robert Rössle Cancer Hospital, Charité University Medicine Berlin, during December 2005 until March 2007 for the first or for a further time were enrolled (Tables 1–3). Histological tumor staging was performed by routine pathology. Patients data describing the histopathological characterization of the tumor (including tumor infiltration, lymph node status, metastasis, grading, lymphatic vessels infiltration, blood vessels infiltration, and residual tumor), documentation of the therapy, and data on survival were available from our tumor bank.

Table 2. Rectal Cancer Patient Characteristics and S100A4 Transcript Levels

		Rectal cancer patients					
		Day of diagnosis			During follow-up		
	Tumor-free volunteers	Newly diagnosed primary tumor without metastasis	Newly diagnosed primary tumor with synchronous metastasis	Newly diagnosed metachronous metastasis	Follow-up without metastasis	Follow-up with metastasis	All
Blood samples, *n*	51	40	11	8	116	15	190
UICC I, %		43	0	67	62	27	51
UICC II, %		32	0	11	19	20	21
UICC III, %		25	0	22	14	53	19
UICC IV, %		0	100	0	5	0	9
Adjuvant therapy		16/40	—	6/8	73/116	10/15	105/190
Follow-up, median, days		762	794	751	705	744	713
Age, median (range), years	60 (27–86)	67 (23–86)	65 (62–73)	70 (53–76)	67 (23–85)	66 (52–76)	67 (23–86)
Sex, male/female	37/14	25/15	9/2	7/1	83/33	8/7	132/58
S100A4 mRNA expression, % calibrator [median]	0.219	0.4375	0.474	0.319	0.3645	0.563	0.38
P, vs tumor-free volunteers		<0.0001	0.0054	0.0335	<0.0001	0.0017	<0.0001

Table 3. Gastric Cancer Patient Characteristics and S100A4 Transcript Levels

		Gastric cancer patient samples					
		Day of diagnosis			During follow-up		
	Tumor-free volunteers	Newly diagnosed primary tumor without metastasis	Newly diagnosed primary tumor with synchronous metastasis	Newly diagnosed metachronous metastasis	Follow-up without metastasis	Follow-up with metastasis	All
Blood samples, *n*	51	11	10	6	60	4	91
UICC I, %		45	0	50	50	0	45
UICC II, %		27	0	0	30	50	25
UICC III, %		18	0	17	17	25	15
UICC IV, %		10	100	33	3	25	15
Adjuvant therapy		1/11	—	2/6	0	0	3/91
Follow up, median, days		693	111	62	735	590	712
Age, median (range), years	60 (27–86)	63 (42–79)	63 (57–77)	71 (38–83)	63 (40–86)	63 (49–74)	63 (38–86)
Sex, male/female	37/14	9/2	9/1	5/1	40/20	4/0	67/24
S100A4 mRNA expression, % calibrator [median]	0.219	0.509	0.5075	0.632	0.384	0.5635	0.411
P, vs tumor-free volunteers		<0.0001	0.005	0.0032	<0.0001	0.0188	<0.0001

Classification of Patients with Newly Diagnosed Disease

Primary tumor of the colon [Union for International Cancer Control (UICC) stage I: T1 to 2N0M0, $n = 6$; II: T3 to 4N0M0, $n = 5$; III: T1 to 4N0 to 1M0, $n = 2$], the rectum (I: T1 to 2N0M0, $n = 17$; II: T3 to 4N0M0, $n = 13$; III: T1 to 4N0 to 1M0, $n = 10$), or the stomach (I: T1 to 2N0 to 1M0, $n = 5$; II: T1 to 3N0 to 2M0, $n = 3$; III: T2 to 4N0 to 2M0, $n = 2$; IV: T1 to 4N1 to 3M0, $n = 1$); three of these 64 patients without metastasis with a primary tumor developed later distant metastases; primary colon: ($n = 10$), rectal ($n = 11$) (both UICC stage IV: T1 to 4N0 to 1M1), or gastric tumor (IV: T4N1 to 3M1, $n = 10$), together with synchronous metastases; metachronous metastases after R0 surgery of primary colon (UICC stage II: T3 to 4N0M0, $n = 3$; III: T1 to 4N0 to 1M0, $n = 2$), rectal (I: T1 to 2N0M0, $n = 5$; II: T3 to 4N0M0, $n = 1$; III: T1 to 4N0 to 1M0, $n = 2$), or gastric cancer (I: T1 to 2N0 to 1M0, $n = 3$; III: T2 to 4N0 to 2M0, $n = 1$; IV: T1 to 4N1 to 3M0, $n = 2$).

Blood samples were taken on the day of diagnosis.

Classification of Patients with Newly Diagnosed Disease in a Test Set and Validation Set

Colorectal cancer patients with newly diagnosed disease were grouped in accordance with their chronological examination and initial blood taking for evaluation of the diagnostic value of S100A4 transcript determination.

Patients (with tumor, with tumor and synchronous metastasis, and with metachronous metastasis) who were analyzed first constituted the test set ($n = 44$) for calculation of the optimal S100A4 mRNA cut-off with regard to sensitivity and specificity.

Patients who were examined in the following constituted the validation set ($n = 43$). Evaluation of S100A4 transcripts was based on the cut-off from the test set.

The cohort of gastric cancer patients was not split because of the restricted number of samples ($n = 27$).

Classification of Follow-Up Patients

We included follow-up patients who visited the physician for follow-up examinations after colon, rectal, or gastric cancer R0 surgery; patients who were deemed to be tumor-free and metastasis-free; and patients who were deemed to be tumor-free but who developed metastases during follow-up.

Blood samples were taken in a median of 1726, 1055, and 944 days after primary diagnosis (median follow-up after blood taking for colon, rectal, and gastric cancer: 742, 705, and 735 days, respectively). Comprehensive lists of all patients samples and their characteristics are provided in Tables 1, 2, and 3.

In general, patients were not treated with chemotherapy or surgery within the last 2 weeks before taking the blood sample. However, for patients with locally advanced rectal cancer treated with neoadjuvant short-course radiation the period between the end of the treatment and taking the blood sample was approximately 3 to 6 days. Surgery was performed within 1 week for the short-course patients after the end of the treatment, but within 4 to 6 weeks for chemoradiation patients.

Several studies report the involvement of S100A4 in nonmalignant diseases, such as inflammation and heart diseases.[35–37] Therefore, patients with active inflammation processes, with coronary heart disease, or who had recently a heart attack were excluded. Patients who had a primary tumor of another entity in history or who developed a second primary tumor during follow-up were also excluded from this analysis.

Collection of Samples

Altogether, 466 blood samples of 361 consecutive tumor patients were examined, with 185, 190, and 91 of colon, rectal, and gastric cancer samples, respectively. Blood was collected daily from hospitalized patients and from the outpatient care. Blood samples were taken at the day of diagnosis from patients newly diagnosed with a primary tumor without synchronous metastases, a primary tumor with synchronous metastasis, or with metachronous metastases. These data mainly served for evaluation of the diagnostic value of S100A4 mRNA. Blood samples from the cohort of follow-up patients were taken exclusively during the follow-up. Because of the follow-up schedule, some patients visited the clinic more than once in the observed time period. There-

fore, more than one blood sample was collected for some patients especially in the follow-up. Furthermore, we also analyzed corresponding samples of seven patients with locally advanced rectal cancer, before and after neoadjuvant treatment.

Blood specimens were obtained with informed written consent. In accordance to the International Conference on Harmonisation, patients' anonymity and confidentiality were preserved. In addition, blood samples of 51 tumor-free volunteers, collected in two independent cohorts, were analyzed. The whole procedure was approved by the local IRB.

Plasma Preparation

Plasma was separated as described by Fleischhacker et al[38] Briefly, 5 ml of cooled EDTA-treated blood was centrifuged at 1300 rpm for 10 minutes at 10°C. The plasma supernatant was again centrifuged at 2500 rpm for 15 minutes and 4°C to remove all cell debris. Plasma samples were stored at −80°C. Samples were blinded so that neither tumor entity nor disease stage was disclosed during analysis.

RNA and Quantitative Real-Time RT-PCR

Total RNA was isolated from plasma by using the High Pure Viral RNA kit according to the recommendations of the manufacturer (Roche Diagnostics, Mannheim, Germany), and concentration of this RNA was measured (2100 Bioanalyzer, Agilent, Santa Clara, CA). Quantitative real-time RT-PCR was carried out as previously described.[29] S100A4 expression analysis was performed based on the hybridization probe detection format using amplicon-specific hybridization probes with the LightCycler system 2.0 (Roche Diagnostics). The following primers (synthesis BioTeZ, Berlin) and probes (synthesis TIB MOLBIOL, Berlin) were used to generate the 124-bp amplicon of S100A4: forward-primer 5′-GAGCTGCCCAGCTTCTTG-3′, reverse-primer 5′-TGCAGGACAGGAAGACACAG-3′, FITC-probe 5′-TGATGAGCAACTTGGACAGCAACA-3′, LCRed640-probe 5′-GACAACGAGGTGGACTTCCAAGAGT-3′. PCR was carried out as follows: 30 seconds at 95°C, 45 times (10 seconds at 95°C, 10 seconds at 60°C, and 10 seconds at 72°C), melting curve 40°C to 95°C. For calibration, we used total RNA from the S100A4 expressing colon carcinoma cell line Colo205. This calibrator RNA was used in serial dilutions for generating a standard curve in each quantitative PCR run. Finally, the S100A4 mRNA expression of a blood sample is given as percentage of the S100A4 mRNA expression of a defined calibrator sample, which was set 100%. Each sample was run in duplicate. Mean values are provided.

There is a large body of papers, recommending how to do normalization *in vitro*, *in vivo*, and in studies analyzing interindividual samples. The use of housekeeping genes, however, is very controversially discussed, particularly when evaluating interindividual differences, as exemplified for the housekeeping genes β-actin and GAPDH.[39,40] Furthermore, not only the variability of the housekeeping genes but also the magnitude of their expression should be considered, and ideally expressed at the same order of magnitude as the gene of interest. Thus the use of total RNA, instead of using housekeeping genes, is recommended for normalization thereby excluding the unknown variable of differentially expressed housekeeping genes in different individuals. To avoid misinterpretation, we followed these guidelines and performed normalization against total RNA.

Statistical Analysis

Results are expressed as median (range) or frequencies (%). After proof of the distribution for normality, differences between the regarded groups in terms of S100A4 transcript levels in plasma were tested by using nonparametric Wilcoxon–Mann–Whitney tests (eg, comparing tumor-free volunteers to patients with tumors, to patients harboring tumors and synchronous metastases, to patients with metachronous metastases, and to follow-up patients without or with metastases). Likewise, comparison of S100A4 in plasma of patients with tumors versus patients with tumors and metastases, or for follow-up patients without versus with metastases, were carried out using this test. In the case of small samples, greater differences in sample sizes, large but unbalanced groups, data sets containing ties, or sparse data, tests were carried out in an exact version. We considered $P < 0.05$ to be significant. Accounts for multiple comparisons were carried out using the sequentially rejective Bonferroni–Holm procedure.[41] All numerical calculations were performed with SPSS (version 18; SPSS Inc, Cary, NC).

To define the diagnostic value of S100A4 transcripts in plasma, a cut-off value of S100A4 sensitivity and specificity were calculated with a fourfold table for colorectal cancer (test and validation sets), rectal cancer, colon cancer, and gastric cancer patients, who were newly diagnosed with a primary tumor without or with synchronous metastases, and for patients who had already undergone R0 surgery of the primary cancer and were newly diagnosed with metachronous metastases compared with the blood samples of 51 tumor-free volunteers.

For DFS in all follow-up patients, Kaplan–Meier curves in combination with log rank test were used. The cut-off value of S100A4 was the median of S100A4 of the entire follow-up groups (metastasized together with nonmetastasized) of each entity. Calculations were performed with SPSS.

Results

S100A4 Transcripts in Tumor-Free Volunteers

First, we defined the basal S100A4 transcript level in human plasma. We analyzed blood of two independent cohorts of tumor-free volunteers from Charité Campus Berlin-Buch ($n = 36$) and from Charité Campus Virchow-Klinikum ($n = 15$) for S100A4 (Figure 1). S100A4 transcripts were detectable in all samples, and re-

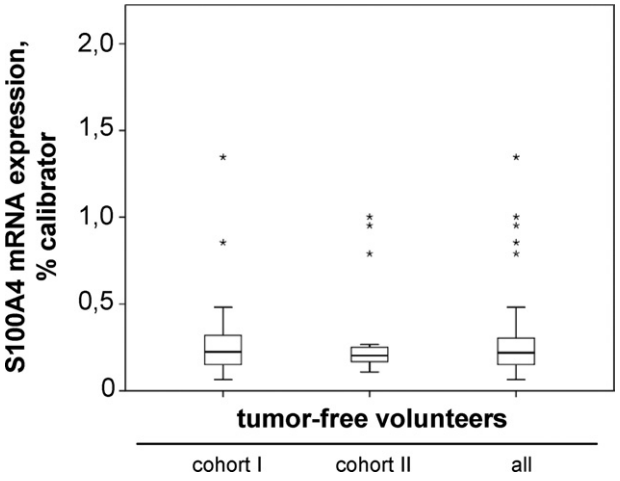

Figure 1. S100A4 transcripts in plasma of tumor-free volunteers. Box plot analysis, based on quantitative real-time RT-PCR (Tables 1–3). No different S100A4 transcript levels were found in two independently analyzed cohorts of tumor-free volunteers.

mained almost unchanged when determined repeatedly in monthly intervals in the same tumor-free individuals. Because the S100A4 levels were not significantly different in both cohorts ($P = 0.9259$; cohort I: median 0.224 S100A4 mRNA expression, % calibrator; cohort II: median 0.203 S100A4 mRNA expression, % calibrator), we combined them for all subsequent analyses (median 0.219 S100A4 mRNA expression, % calibrator) (Figure 1). Significant variations of S100A4 levels due to the age and sex of the individuals were not observed. S100A4 levels showed neither circadian dependence nor dependence on food intake.

S100A4 Transcripts in Plasma Discriminate Cancer Patients from Tumor-Free Volunteers

Next, we assessed S100A4 transcript plasma levels for discrimination of cancer patients and tumor-free individuals (Tables 1–3; Figure 2). We began with colorectal cancer patients' blood ($n = 375$, Tables 1 and 2). S100A4 mRNA was detected in all plasma samples. We determined a statistically significant difference for all colorectal cancer patients' samples compared to the tumor-free volunteers ($P < 0.0001$). This also held true when subclassifying the colorectal cancer patient's samples into a colon ($n = 185$; Table 1; $P < 0.0001$) and a rectal cancer subcohort ($n = 190$; Table 2; $P < 0.0001$). In addition, we analyzed blood from gastric cancer patients and found significantly higher S100A4 transcript levels compared with those in the tumor-free individuals ($n = 91$; Table 3; $P < 0.0001$).

No differences in S100A4 levels were found when comparing colorectal, colon, or rectal cancer patient's samples with those of gastric cancer patients. Interestingly, significantly higher S100A4 levels were determined in colon cancer samples when compared with rectal cancer samples ($P = 0.041$). Thus, we subclassified these entities in the following analyses.

S100A4 Transcripts in Plasma for Improved Diagnosis of Cancer Patients Newly Diagnosed with a Primary Tumor Without or With Distant Metastases

In a next step, patients were classified according to disease stages: patients who were newly diagnosed with a primary tumor, patients who were newly diagnosed with a primary tumor together with synchronous metastases, and patients who already underwent R0 surgery of the primary cancer and were newly diagnosed with metachronous metastases. Remarkably, colon (Table 1, Figure 3A), rectal (Table 2, Figure 3B), and gastric cancer patients (Table 3, Figure 3C) of each of these groups demonstrated significantly higher S100A4 levels than the tumor-free volunteers. However, S100A4 levels were found to be independent of UICC stages within the patients' group with a primary tumor only, as observed for all entities. Increased S100A4 levels were determined for colon cancer patients with a primary tumor and synchronous metastases compared to patients with a primary tumor only ($P = 0.0053$; Figure 3A). Furthermore, S100A4 levels were higher in colon cancer patients newly diagnosed with metachronous metastases compared to those with a primary tumor only ($P = 0.0022$, Figure 3A). Thus, the determination of S100A4 transcript plasma levels points preferentially to an improved diagnosis for distant metastases of colon cancer patients. No significantly different S100A4 levels were found for rectal and gastric cancer patients with metastasis, neither synchronously nor metachronously, compared to patients with only the primary tumor (Figures 3B and 3C, respectively). In addition, no significant differences were found when comparing S100A4 levels to patients' characteristics such as age and sex in any of these groups and for any of each entities.

To evaluate the diagnostic power of this blood-based S100A4 mRNA assay, we analyzed sensitivity and specificity for patients who were newly diagnosed with a pri-

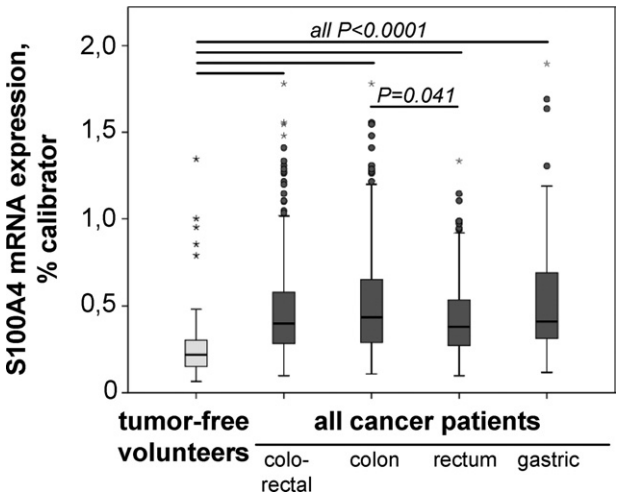

Figure 2. S100A4 transcripts in plasma discriminate cancer patients from tumor-free volunteers. Box plot analysis, based on quantitative real-time RT-PCR (Tables 1–3). All patient cohorts with colorectal, colon, rectal, or gastric cancer expressed significantly higher S100A4 transcript levels than healthy volunteers.

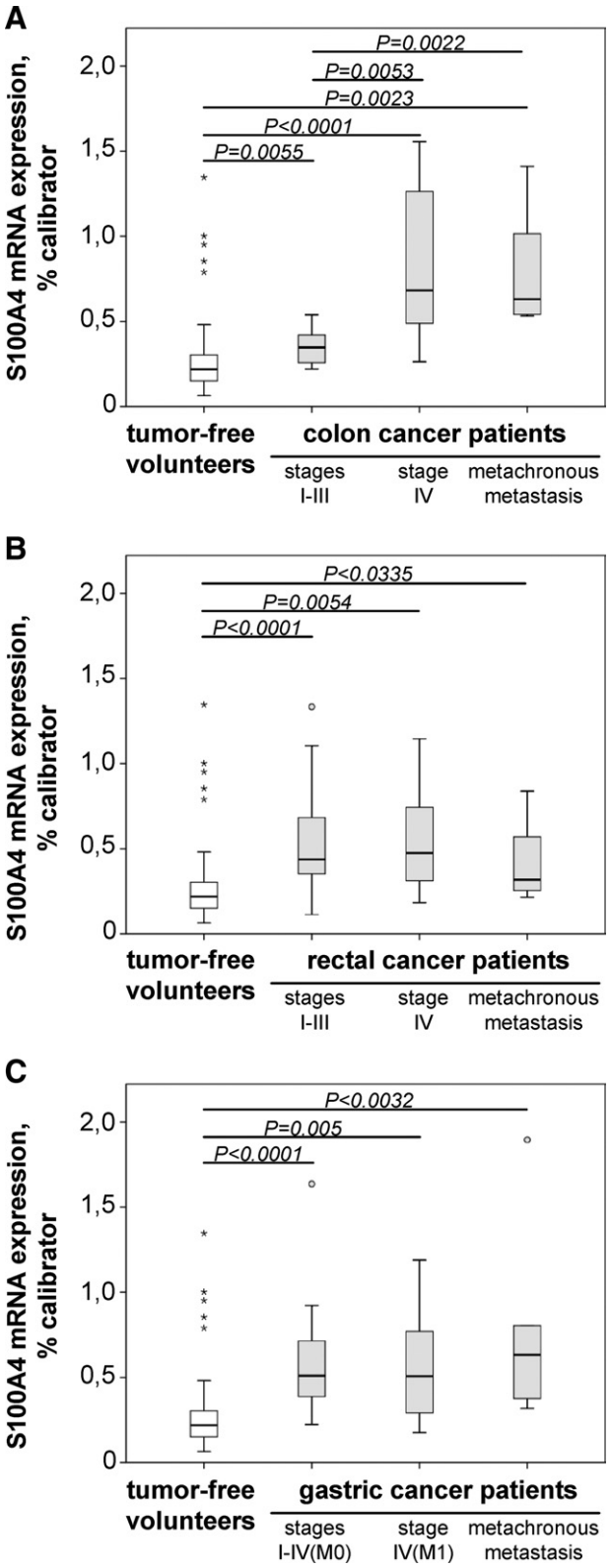

Figure 3. S100A4 transcripts in plasma of patients newly diagnosed with a primary tumor without or with synchronous metastasis, or with metachronous metastasis. Box plot analysis, based on quantitative real-time RT-PCR (Tables 1–3). All patients' subcohorts with colon (**A**), rectal (**B**), and gastric (**C**) cancer expressed significantly higher S100A4 transcript levels than healthy volunteers. Significantly different S100A4 levels were also found for colon (**A**) patients of stages I–III versus IV, and for colon cancer patients without and with metachronous metastasis (**A**).

Table 4. Sensitivity and Specificity for S100A4 Transcript Levels in Plasma of Colon, Rectal, and Gastric Cancer Patients with Newly Diagnosed Primary Tumors, without and with Metastasis

Cancer	Samples *n*	Cut-off S100A4 mRNA expression, % calibrator, [median]	Sensitivity %	Specificity %
Colon	28	0.232	96	59
Rectal	59	0.346	74	82
Gastric	27	0.289	90	71

mary tumor without or with synchronous metastases, and for patients who already underwent R0 surgery of the primary cancer and were newly diagnosed with metachronous metastases. We began with a test set of colorectal cancer patients ($n = 44$), with a sensitivity of 73% and a specificity of 82% (cut-off 0.358 S100A4 mRNA expression, % calibrator). This cut-off was then used for the validation set of colorectal cancer patients ($n = 43$), resulting in a sensitivity of 68% and a specificity of 82%. When combining all colorectal cancer patients, sensitivity was 71% and specificity was 82%.

Next, we used cut-off values specific for colon or for rectal cancer patients, and determined sensitivities of 96% and 74% and specificities of 59% and 82% for colon and rectal cancer patients, respectively (Table 4). For gastric cancer patients, we determined a sensitivity of 90% and a specificity of 71% (cut-off 0.289 S100A4 mRNA expression, % calibrator) (Table 4). Based on these analyses, the quantitative determination of S100A4 in human plasma contributes to the identification of patients suffering of primary colon, rectal, and gastric cancer.

S100A4 Transcripts in Plasma for Improved Prognosis of Follow-Up Cancer Patients

We also analyzed S100A4 in plasma of follow-up colon, rectal, and gastric cancer patients (blood samples were taken in a median of 1726, 1055, and 944 days after primary diagnosis; median follow-up after blood taking was 742, 705, and 735 days, respectively). Although all patients underwent R0 surgery, the patients' groups of each entity showed increased S100A4 levels compared to tumor-free volunteers (Tables 1–3; Figure 4). Some patients thereof developed distant metastases during the follow-up, but after the S100A4 transcript determination. Interestingly, patients with metastases had shown a tendency toward higher S100A4 levels than patients without metastasis. Comparing follow-up patients with metastasis to those without, in accordance to the sequentially rejective test procedure, the tests show multiple significance for patients with rectal cancer ($P = 0.038$; Figure 4B), and strong tendencies for patients with colon cancer ($P = 0.0504$; Figure 4A) and gastric cancer ($P = 0.0595$; Figure 4C). Thus, S100A4 transcript levels in plasma determined during the follow-up and independent from blood samples taken at the time of the initial diagnosis might be of prognostic value for metastases formation of follow-up patients.

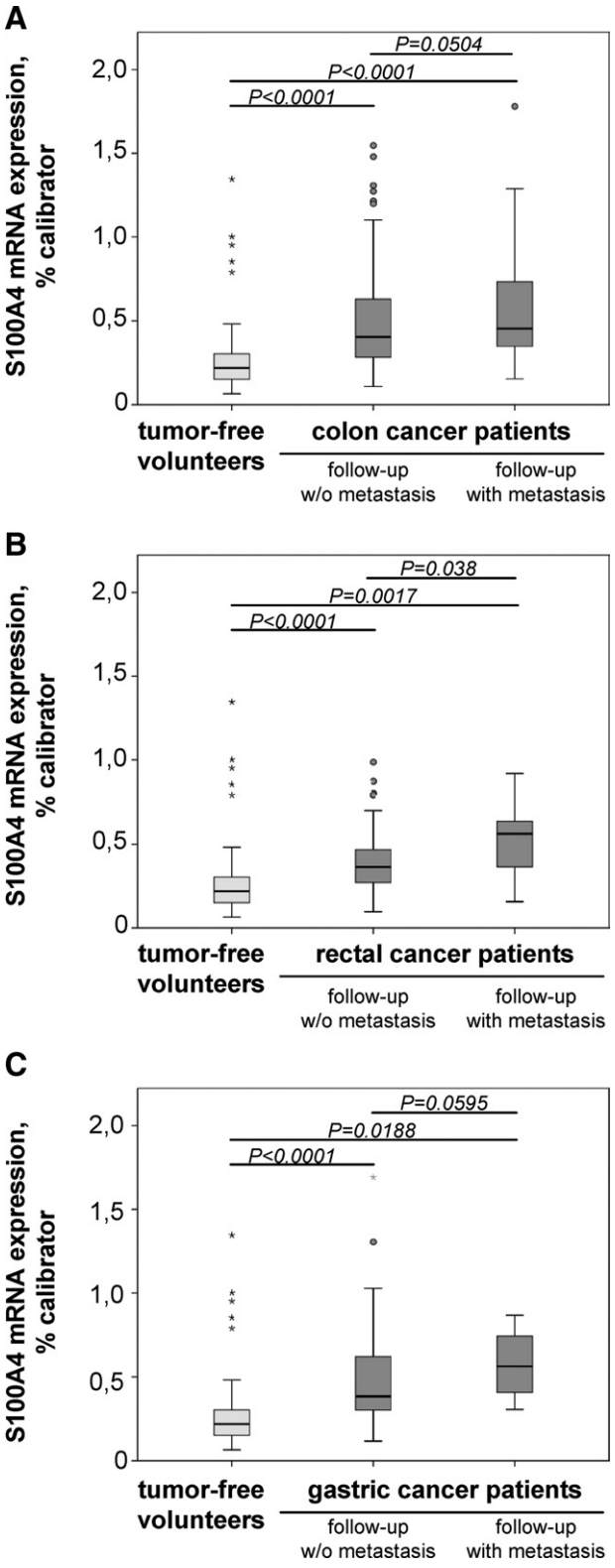

Figure 4. S100A4 transcripts in plasma of follow-up cancer patients without or with distant metastases. Box plot analysis, based on quantitative real-time RT-PCR (Tables 1–3). All follow-up patient subcohorts with nonmetastasized or metastasized colon (**A**), rectal (**B**), and gastric (**C**) cancer expressed significantly higher S100A4 transcript levels than healthy volunteers. Significantly different S100A4 levels were also found for rectal (**B**) follow-up patients without versus with metastasis; higher S100A4 levels were determined in patients with metastasized colon (**A**) and gastric (**C**) cancer compared with patients without metastasis.

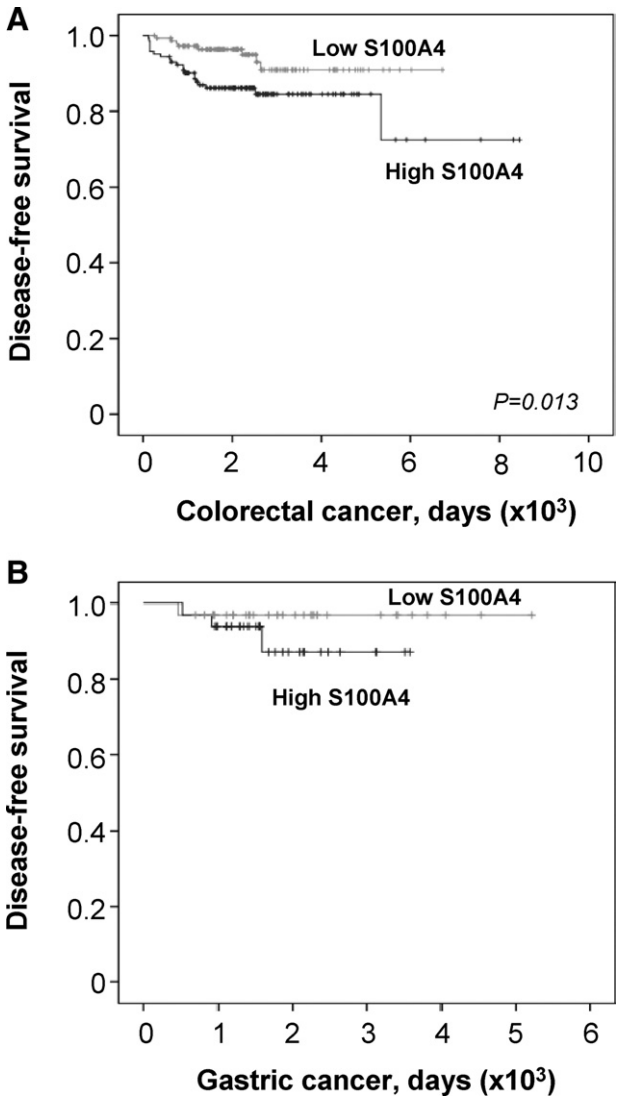

Figure 5. S100A4 transcripts in plasma of follow-up cancer patients and DFS. **A:** Kaplan–Meier analysis for DFS of all follow-up colorectal cancer patients (n = 288, Tables 1 and 2). Based on the median (0.387 S100A4 mRNA expression, % calibrator), all follow-up patients were subclassified into S100A4 transcript low (below median; n = 145) and high (above median; n = 143) expressors. DFS was significantly decreased in patients with high S100A4 transcript levels (P = 0.013). **B:** Kaplan-Meier analysis for DFS of all follow-up gastric cancer patients (n = 64, Table 3). Based on the median (0.39 S100A4 mRNA expression, % calibrator), all follow-up patients were subclassified into S100A4 transcript low (below median; n = 32) and high (above median; n = 32) expressors.

S100A4 Transcripts in Plasma of Follow-Up Cancer Patients and DFS

We also investigated the S100A4 transcript levels in plasma of all follow-up patients in the context of DFS. DFS was defined as survival, free of the events of recurrence and metastasis formation. Interestingly, unfavorable DFS was observed for S100A4 high expressing colorectal as well as gastric cancer patients (Figure 5). In particular, DFS was significantly reduced in follow-up patients with high S100A4 levels who had colorectal cancer (P = 0.013, log-rank test; Figure 5A). The cut-off value of S100A4 was the median of S100A4 calculated for the

entire follow-up group of each entity (colorectal cancer 0.387 S100A4 mRNA expression, % calibrator; gastric cancer 0.39 S100A4 mRNA expression, % calibrator). Thus, the transcript levels of S100A4 are not only of diagnostic but also of prognostic value.

Discussion

Here we report for the first time the transcript determination of the metastasis inducer S100A4 in human plasma. The overriding goal of this study was to define the diagnostic and prognostic power of S100A4 transcripts in blood samples from colon, rectal, and gastric cancer patients. These analyses are based on data, including our own, that show the diagnostic and prognostic value of S100A4 when detected in solid tumors.[20–34]

We determined S100A4 mRNA quantitatively in human plasma, and found higher S100A4 levels in cancer patients than in tumor-free volunteers, with sensitivities of 96%, 74%, 90%, and specificities 59%, 82%, 71%, for colon, rectal, and gastric cancer patients, respectively. S100A4 mRNA detection is applicable and might support screening for occult tumors and/or metastasis in healthy populations. A small study reported detection of S100A4 mRNA in serum of breast cancer patients; however, not using gene-specific quantitative real time RT-PCR.[42] The use of S100A4 protein as tumor marker in human blood, however, was controversially evaluated.[43,44]

We have shown here the diagnostic relevance of S100A4 transcript plasma levels even for early disease stages before the event of metastasis, given that the S100A4 levels in patients with a nonmetastasized primary tumor were higher than those of the tumor-free control groups of each entity analyzed. We did not find different S100A4 plasma levels in the tumor stages without distant metastasis. This finding is confirmed by former studies analyzing S100A4 mRNA levels in solid tissues of colon cancer patients.[29,45] We and others determined an independence of S100A4 mRNA expression determined in colon tumor tissues of stages I, II, and III.

However, significantly higher S100A4 plasma levels were determined at the day of the first diagnosis in colon cancer patients with synchronous metastasis. S100A4 transcript levels might also contribute to identify a metastasized disease stage. This finding was also revealed by earlier studies performed with colon cancer tissues.[29,45] In addition, S100A4 mRNA expression is of prognostic value for the development of metachronous distant metastases when determined in tissues of the nonmetastasized primary tumor.[29] However, only three of all newly diagnosed 64 nonmetastasized primary tumors, with blood samples taken at the day of the first diagnosis, have so far developed distant metastases metachronously. Therefore, the prognostic impact of S100A4 transcripts in plasma for the individual metachronous metastasis risk for patients newly diagnosed with a primary tumor only cannot be so far. Longer follow-up times and higher numbers of blood samples might confirm the previous knowledge by using the new plasma-based technology. Taken together, S100A4 levels in plasma of newly diagnosed patients were found to be independent of UICC stages I, II, and III, but dependent on metastasis.

The transcriptional regulation of S100A4 might be decisive for the early onset of S100A4 mRNA expression during tumor progression. For instance, S100A4 was identified as a transcriptional target of β-catenin.[29] Mutant β-catenin acts in a dominant fashion in colon cancer, and is found at the very early steps during tumor progression inducing a gene expression pattern for invasion and metastasis. Consequently, β-catenin target genes such as S100A4 or further metastasis-associated genes such as in matrix metalloproteinases are up-regulated already in the early tumor stages. Furthermore, ErbB2 activates S100A4 expression already during the epithelial–mesenchymal transition via the Ras/Raf/Mek/Erk1/2 signaling pathway, and S100A4 expression might also be induced early during tumor progression by $\alpha 6\beta 4$ integrin via NFAT5.[46–48] Furthermore, today it is widely acknowledged that the metastatic capacity of a cell is already determined at a very early stage of tumor progression.[49,50] Therefore, the completion of the linear adenoma–carcinoma sequence finally leading to metastasis does not necessarily reflect the situation at the molecular level. This is supported by, microarray analyses with colorectal cancer tissues that show similar patterns of metastasizing cells and the early primary tumor from which they originated.[51] In addition, the current TNM staging system is critically discussed, particularly for colorectal cancer, pointing to the need for new factors, either morphological or molecular, that could more precisely stratify patients into different risk categories.[52]

Furthermore, we determined prospectively S100A4 transcript plasma levels in all 352 follow-up cases. Interestingly, follow-up patients who subsequently developed distant metastases expressed higher S100A4 levels than follow-up patients without metastasis. Therefore, metastases formation correlated with high S100A4 levels in plasma of colon, rectal, and gastric cancer patients even when determined after surgery during the follow-up. This remarkable finding points to the prognostic value of S100A4 with respect to disease course (metastasis) for follow-up patients, who account with 74% for the largest groups of all analyzed patients. Notably, DFS was also lowered for follow-up patients with high S100A4 transcript plasma levels. This finding supports the role of S100A4 also for patients' survival. Moreover, it underlines the usefulness of this blood-based assay for monitoring purposes.

We also analyzed the influence of tumor-related neoadjuvant treatments on S100A4 levels. This analysis was possible in locally advanced rectal cancer. We analyzed the 40 primary rectal cancer cases (stages I to III) before and after neoadjuvant treatment. We found a clear but not significant decrease of S100A4 transcripts when comparing corresponding samples before and after treatment originating from the same patients. This decreased S100A4 expression was independent of the treatment schedule (short-course radiotherapy or chemoradiation), but might point to a therapy-induced modulation of S100A4 levels. This would provide the rationale for a S100A4-based monitoring of therapy response. However, the diverse intervals between neoadjuvant treat-

ment and taking the blood sample (at least 3 days for short-course radiation and at most 6 weeks for chemoradiation) might account for the non-significant decrease of S100A4 transcripts. Additional studies will be necessary to elucidate the potential of S100A4 transcripts in blood for prediction and monitoring of therapy response. For colon cancer, there is no recommendation of neoadjuvant treatment, and the impact of neoadjuvant therapy in gastric cancer is still controversial.

Circulating nucleic acids including transcripts in plasma or serum of patients have been evaluated as diagnostic, prognostic, and predictive markers for solid neoplasias.[8–10] For gastric cancer, osteopontin plasma levels have been shown to be significantly associated with invasion and patient survival.[53] hTERT and MUC1 mRNAs were found to be overexpressed in the plasma of gastric cancer patients but were not detectable in plasma of healthy volunteers.[54]

For colorectal cancer, hTERT(N) mRNA in plasma clearly differentiates between healthy and colorectal cancer patients.[55] Elevated levels of epithelial tumor RNA (CK19, CEA) and thymidylate synthase transcripts in plasma of colon cancer patients are associated with advanced stages and poor prognosis.[56–59] The prognostic value of LISCH7 transcripts, a gene that is regulated by p53 and overexpressed in colon cancer metastasis development, was shown in plasma of colon cancer patients.[60]

Interestingly, increased levels of β-catenin, which plays a crucial role for Wnt pathway signaling and colorectal pathogenesis, have been determined in plasma and were correlated with tumor stage.[61] Activation of the Wnt/β-catenin pathway is frequently observed in colorectal cancers, and β-catenin gene mutations have been described as early and critical steps in the genesis of the disease. We demonstrated previously that β-catenin/TCF directly regulates the expression of S100A4, and that gain-of-function β-catenin acts in a dominant manner.[29,62] Activation of Wnt/β-catenin signaling, together with high S100A4 expression, was also reported for gastric cancer.[33,34]

In summary, we developed a noninvasive, reliable, and simple method for quantitative determination of S100A4 transcripts in human plasma that allows for routine clinical application. We demonstrated the diagnostic and prognostic potential of S100A4 transcripts in plasma of colon, rectal, and gastric cancer patients for early defining cancer staging and patients' risk for metastasis. Combinatorial detection of relevant transcripts might even enhance diagnosis, prognosis, or prediction. Additional S100 genes, such as S100A8 and S100A9, recently identified as protein markers in colorectal cancer patients' blood, or transcripts already described in the context of gastrointestinal cancer, might add to a more comprehensive and thus personalized approach.[63] Our data on the metastasis inducer S100A4 mRNA, which is now detectable in patients' plasma, might therefore contribute to personalization of initial and additional therapy that could strongly enhance patient care.

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