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Advances in Brief

S100 Family Members and Trypsinogens Are Predictors of Distant Metastasis and Survival in Early-Stage Non-Small Cell Lung Cancer

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Abstract

Distant metastasis is the predominant cause of death in early-stage non-small cell lung cancer (NSCLC). Currently, it is impossible to predict the occurrence of metastasis at early stages and thereby separate patients who could be cured by surgical resection alone from patients who would benefit from additional chemotherapy. In this study, we applied a comparative microarray approach to identify gene expression differences between early-stage NSCLC patients whose cancer ultimately did or did not metastasize during the course of their disease. Transcriptional profiling of 82 microarrays from two patient groups revealed differential expression of several gene families including known predictors of metastasis (*e.g.*, matrix metalloproteinases). In addition, we found S100P, S100A2, trypsinogen C (TRY6), and trypsinogen IVb (PRSS3) to be overexpressed in tumors that metastasized during the course of the disease. In a third group of 42 patients, we confirmed the induction of S100 proteins and trypsinogens in metastasizing tumors and its significant correlation with survival by real-time quantitative reverse transcription-PCR. Overexpression of S100A2, S100P, or PRSS3 in NSCLC cell cultures led to increased transendothelial migration, corroborating the role of S100A2, S100P, and PRSS3 in the metastatic process. Taken together, we provide evidence that expression of S100 proteins and trypsinogens is associated with metastasis and predicts survival in early stages of NSCLC. For the first time, this implicates a role of S100 proteins and trypsinogens in the metastatic process of early-stage NSCLC.

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of tumor-related death. Distant metastasis is the most frequent reason for NSCLC lethality. Despite recent progress, the molecular mechanisms underlying metastasis have not been solved in detail. Several investigators have analyzed the involvement of individual genes (1, 2), gene expression differences in cell lines (3), and gene expression differences between primary tumors and normal lung tissue (4). These studies help to decipher biological differences in these systems but do not aid in the definition of prognostic parameters at diagnosis. Only gene expression differences in primary tumors before the onset of metastasis allow the development of molecular biology-based therapy decisions (5). Sets of genes predictive for survival prognosis of NSCLC patients have been defined, based on microarray experiments (6). For example, microarray analyses uncovered a histologically nondistinct tumor subgroup associated with refractory disease (7, 8). In addition, gene expression changes associate with disease-free survival (6, 9), and transcriptional profiling studies have distinguished novel histological NSCLC subtypes (10). However, a distinct study comparing expression profiles of primary tumors at the stage of diagnosis and identifying patterns underlying metastasis is still lacking. These data could reveal deeper insights into the molecular biology of the metastatic process and would have clinical implications. It could be possible to identify patients who would benefit from adjuvant chemotherapy after resection of the primary tumor. We performed transcriptional profiling using oligonucleotide arrays of early-stage NSCLC tumors that did or did not lead to distant metastasis after prolonged follow-up. We identified several groups of genes including S100 proteins and trypsinogens that predict metastasis and survival at the time of diagnosis and are involved in the metastatic process.

Materials and Methods

Gene Expression Analysis. Primary tumor specimens were obtained at the time of initial surgery for early-stage NSCLC (2). Patient data have been published previously (5, 7). Oligonucleotide microarray (Affymetrix) hybridizations were carried out as described previously (11, 12). We hybridized cRNA obtained from 14 patients (15 arrays) with stage I or II NSCLC (adenocarcinoma and squamous cell carcinoma). In addition, we used published primary data from a second group of 52 patients (67 arrays; Ref. 7). Regulation (Fig. 1*A*) was calculated as the mean of the regulation of the average and of the median expression levels in both groups. In addition, S100A2, S100P, PRSS3, and TRY6 mRNA expression levels were analyzed by quantitative real-time reverse transcription-PCR [RT-PCR (Ref. 5; see Supplementary Fig. 1 for sequences)]. Statistical analyses were carried out using SPSS 11.0. All tests were two-sided, with $P = 0.05$ regarded as significant.

Migration Analysis. Coding sequences of S100P, S100A2, PRSS3, and TRY6 were cloned into expression vector pcDNA3.1(+) harboring enhanced green fluorescent protein (EGFP) and stably transfected into HTB-58 (SK-MES-1). The use of bulk cultures avoided clone-specific effects. Expression was verified by quantitative RT-PCR and Western blotting [anti-EGFP (Clontech) and anti-actin (Sigma)] as well as fluorescence-activated cell-sorting (FACS) analysis. For migration analysis, 5×10^5 cells were plated into Transwell plates precoated with fibronectin. Migration was measured by counting the number of cells that migrated into the lower chamber after 24 h by FACS. Transendothelial migration (13) was detected in the same way, but 2.2×10^5 HMEC-1 endothelial cells were plated into the Transwell plates 48 h before seeding the transfected NSCLC cell cultures.

Further information can be found in the supplementary data.

Results

Identification of Metastasis-Associated Genes by Comparative Microarray Analysis. We used microarray analyses to identify genes predicting the likelihood of early-stage NSCLC tumors to metastasize. For this purpose, tumors resected from patients with stage I or II

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A

UniGene	GenBank	Name	Gene	Regulation
Proteases				
Hs.2399	Z48481	MMP14	**Matrix-Metalloproteinase 1, membrane-type (MT-MMP1)**	18,04
	U66061 / NR_001296	TRY6	**Trypsinogen C**	5,91
Hs.2258	X07820	MMP10	**Matrix-Metalloproteinase 10 (Stromelysin-2)**	3,98
Hs.435699	X71345	PRSS3	**Trypsinogen IVb**	3,49
Hs.368077	L40377	CAP2	**Cytoplasmic Antiproteinase 2 (Protease Inhibitor Serpin B8)**	2,86
S100 proteins				
Hs.413843	M87068	S100A2	**S100A2 (S100L = CaN19)**	4,15
Hs.515713	AI539439	S100A2	**S100A2**	4,09
Hs.2962	AA131149	S100P	**S100P**	4,01
ECM-related proteins & Their Regulators				
Hs.355214	J00124	KRT14	**Keratin 14 (50kD Epidermal Keratin Type I)**	4,68
Hs.432448	M28439	KRT16	**Keratin 16**	4,29
Hs.407506	M13981	INHA	**Inhibin A**	4,02
Hs.158446	AA523313	RBMS3	**RNA-binding protein S3**	2,97
Hs.126248	L41162	COL9A3	**Collagen alpha 3 type IX**	0,37
Signal Transduction Proteins & Ion Channels				
Hs.1076	M19888	SPRR1B	**Small proline-rich protein 1B**	7,24
Hs.301350	U28249	FXYD3	**FXYD containing Ion Transport Regulator 3**	3,76
Hs.350470	AA314825	TFF1	**Trefoil Factor-1**	2,76
Hs.417091	AF052117	CLCN4	**Chloride Channel 4**	2,74
Hs.54506	U49379	DGKE	**Diacylglycerol Kinase epsilon**	2,73
Hs.40499	AB020315	DKK1	**Dickkopf-1**	2,69
Hs.63984	L34058	CDH13	**Cadherin 13**	2,54
Hs.7306	AF056087	SFRP1	**Secreted frizzled-related protein**	0,40
Enzymes				
Hs.83834	M22976	CYB5	**Cytochrome b5**	4,19
Hs.198281	W28740	PK3	**Pyruvate Kinase**	3,46
Hs.575	M74542	ALDH3A1	**Aldehyde dehydrogenase Type III**	3,14
Hs.279916	AF037335	CA12	**Carbonic Anhydrase precursor 12**	2,75
Proteins of Blood, Immune System & Inflammation				
Hs.75431	AI989422	FGG	**Fibrinogen, gamma polypeptide**	6,43
Hs.418062	Y15062	B3GALT3	**UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 3**	3,43
Hs.82120	S77154	NR4A2	**NGFI-B/nur77-related transcription factor**	2,78
Hs.624	M28130	IL8	**Interleukin-8**	2,69
Neuronal Proteins				
Hs.282566	AB020650	ABLIM3	**ABLIM3 actin binding LIM protein family, member 3**	2,85
Hs.75819	D49958	GPM6A	**Membrane Glycoprotein M6**	2,82
Hs.54471	D45399	PDE6H	**cGMP phosphodiesterase, cone-specific**	2,81
Hs.433429	D30036	PITPN	**Phosphatidylinositol transfer protein**	2,64
Cell Adhesion Proteins				
Hs.501990	AA010777	LGALS7	**Galectin-7**	3,79
Hs.232072	AF055580	USH2A	**Usher syndrome type IIa protein**	2,82
Hs.415762	Y12642	E48	**E48 Lymphocyte antigen 6 complex, locus D**	2,54
Other Proteins				
	U93163	MAGE B	**MAGE antigens**	4,24
Hs.255462	AA532495	MSMB	**Microseminoprotein**	3,75
Hs.512581	X06290	LPA	**Apolipoprotein a**	3,44

B

	Microarrays			RT-PCR
	Group 1	Group 2	Total	Group 3
Patients	14	52	**66**	42
NO	5	28	**33**	29
YES	9	24	**33**	13
Chips	15	67	**82**	

C

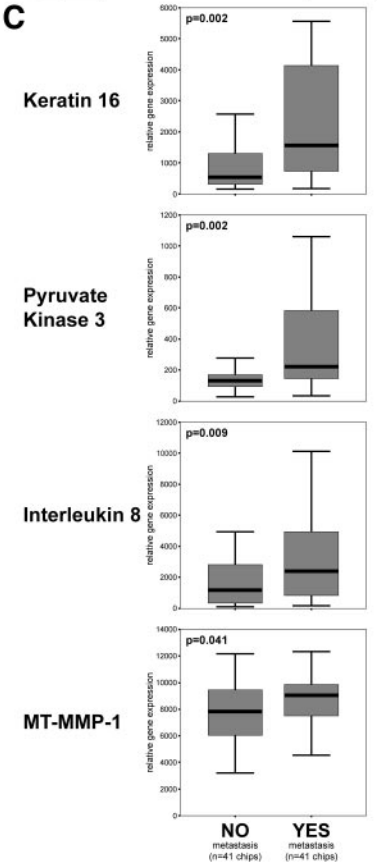

Fig. 1. Thirty nine genes were differentially expressed in NSCLC metastasis. *A*, we identified genes that were differentially expressed in primary tumors from early-stage NSCLC patients who did or did not develop distant metastasis during the course of their disease. Microarray analysis revealed 37 up-regulated and 2 down-regulated genes among more than 12,000 genes ($n = 82$). *B*, in total, three different groups of tumor specimens from early-stage NSCLC patients were used in our analyses (two subsets for microarray hybridizations and one subset for the verification of expression differences by real-time quantitative RT-PCR). *C*, several of the identified genes have previously been described to be involved in NSCLC metastasis, thus providing evidence for the validity of our approach. These were significantly up-regulated in metastasizing tumors (*YES*) compared with nonmetastasizing tumors (*NO*).

NSCLC were shock frozen, and RNA was isolated and hybridized onto HGU95Av2 Affymetrix chips containing more than 12,000 genes. The final data set contained 82 microarrays because we combined our hybridization data with published expression profiles not analyzed for metastasis-associated genes (7). Patients were followed over time for at least 36 months.

In our analyses, we identified 39 genes with differential expression in patients whose cancer metastasized during the course of the disease (YES) and patients whose cancer did not metastasize (NO). Only two genes (collagen COL9A3 and secreted frizzled-related protein SFRP1) were down-regulated in metastasizing tumors, whereas 37 genes were up-regulated in metastasizing tumors. We provide a gene list, in which we added the new UniGene nomenclature in addition to the accession numbers given by Affymetrix and substituted all expressed sequence tags by the recently identified genes (Fig. 1*A*). The identified genes belong to different functional groups *e.g.*, proteases, calcium-binding S100 proteins, extracellular matrix (ECM) proteins, or metabolic enzymes.

Some of the genes identified in our screen have previously been associated with metastasis. The matrix metalloproteinases (MMPs) degrade ECM proteins, an essential step for migration and invasion. MMPs have been linked previously to metastasis in NSCLC (3). In our analyses, we isolated MT-MMP1 (MMP14) and stromelysin-2 (MMP10). In addition, members of the keratin family of intracellular IF proteins were associated with metastasis in NSCLC (14). In contrast, the ECM protein collagen was inhibitory for cell motility and migration (15). We discovered up-regulation of keratin 14 and keratin 16, as well as down-regulation of collagen 9A3. The chemokine interleukin 8 has been associated with metastasis and tumor angiogenesis (16) as well as the metabolic enzyme pyruvate kinase 3 (PK-M2; Ref. 17). We demonstrate significant association of MT-MMP1 (MMP14), keratin 16, pyruvate kinase 3 (PK-M2), and interleukin 8 with the occurrence of metastasis based on analysis of all microarrays ($n = 82$; Fig. 1*C*).

Association of S100 Proteins with Metastasis and Survival in NSCLC. The family of calcium-binding S100 proteins fulfills a broad range of functions in calcium-dependent stimulus response coupling and has not been implicated in the metastatic process in NSCLC. Among the S100 proteins, S100A4 (Mts1) has been linked to metastasis in mammary tumors (18). However, S100A4 expression did not correlate with melanoma progression, in which S100A2 expression was lost (19), indicating distinct roles of the S100 proteins in malignant processes.

In our analyses, two of the regulated genes belonged to the family of S100 proteins (three oligonucleotide sets, S100A2 = S100L, S100P; Fig. 2*A*).

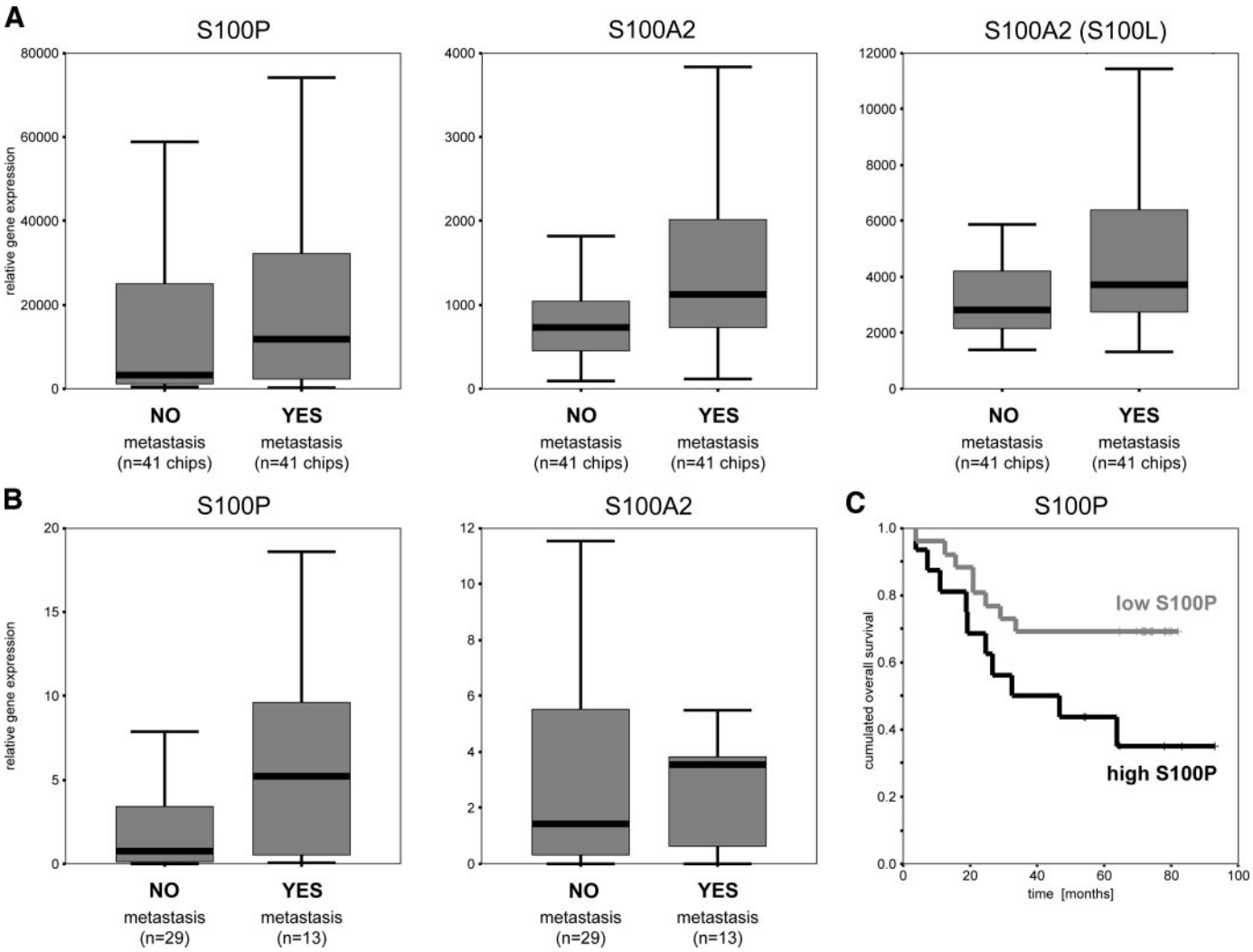

Fig. 2. Association of S100 proteins with metastasis and survival in NSCLC. *A*, induction of S100 protein family members S100P, S100A2 ($P = 0.008$), and S100L ($P = 0.017$) in metastasizing tumors was detected in microarray analysis. In the *box plot*, the *box* indicates the range of 50% of the samples (25–75% quartile) and the median expression, whereas the *outer lines* indicate the range from the 10th percentile to the 90th percentile. *B*, real-time quantitative RT-PCR analysis confirmed the overexpression of S100P and S100A2 in a different group of patients ($n = 42$). *C*, in Kaplan-Meier survival curves of mRNA expression levels determined by real-time quantitative RT-PCR, patients with high expression of S100P had a significantly worse prognosis ($P = 0.046$).

We confirmed differential expression by real-time quantitative RT-PCR in a group of 42 patients that had not been included in the microarray analyses. In this group, we found 3- to 7-fold induction of the median expression in metastasizing *versus* nonmetastasizing tumors and thereby verified the regulation of S100A2 and S100P with a different method (Fig. 2*B*).

The 42 patient samples analyzed by real-time quantitative RT-PCR were divided into high (3-fold above median; $n = 16$) and low ($n = 26$) S100P expressers. Kaplan-Meier analysis revealed a significant benefit in overall survival for the patients with low expression of S100P (Fig. 2*C*; $P = 0.046$). The same trend was observed for S100A2 but was not statistically significant.

Association of Trypsinogens with Metastasis and Survival in NSCLC. The serine protease trypsin and its precursor trypsinogen have been linked to tumor progression by activation of MMPs in pancreatic, gastric or colorectal cancer (20). Our microarray analysis revealed up-regulation of two trypsinogens [trypsinogen IVb (PRSS3) and trypsinogen C (TRY6)] in the metastatic process of NSCLC (Fig. 3*A*). The induction of PRSS3 and TRY6 in metastasizing compared with nonmetastasizing NSCLC tumors was confirmed in the additional subset of patient samples ($n = 42$) by real-time RT-PCR (Fig. 3*B*). In Kaplan-Meier analyses, patients with high expression of either PRSS3 or TRY6 suffered from a substantially worse prognosis than patients expressing low levels of trypsinogens (Fig. 3*C*).

Stimulation of Migration in NSCLC Cell Cultures by S100 Proteins and Trypsinogens. To analyze the role of S100 proteins and trypsinogens in the metastatic process, we studied their effect on cell migration and invasive migration through endothelial cell layers. We established HTB-58 NSCLC cell cultures stably expressing EGFP (control vector) or EGFP fusion constructs of S100P, S100A2, PRSS3, or TRY6. To exclude clone-specific effects, bulk cultures of selected and sorted cells were used after transfection. Overexpression was verified by FACS analysis, real-time RT-PCR, and Western blotting. All cell cultures displayed at least 90% EGFP positivity (Fig. 4*A*). Fluorescence microscopy confirmed distinct localization patterns of the fusion proteins (data not shown). Overexpression at the mRNA level varied between the different cell cultures from 2-fold to 4000-fold, probably reflecting differences in endogenous gene expression or mRNA stability (Fig. 4*B*). However, consistent expression of the EGFP fusion proteins was detected at the protein level by FACS analysis. Western blotting for EGFP demonstrated expression of the EGFP fusion proteins in stable cell cultures. A nonspecific band

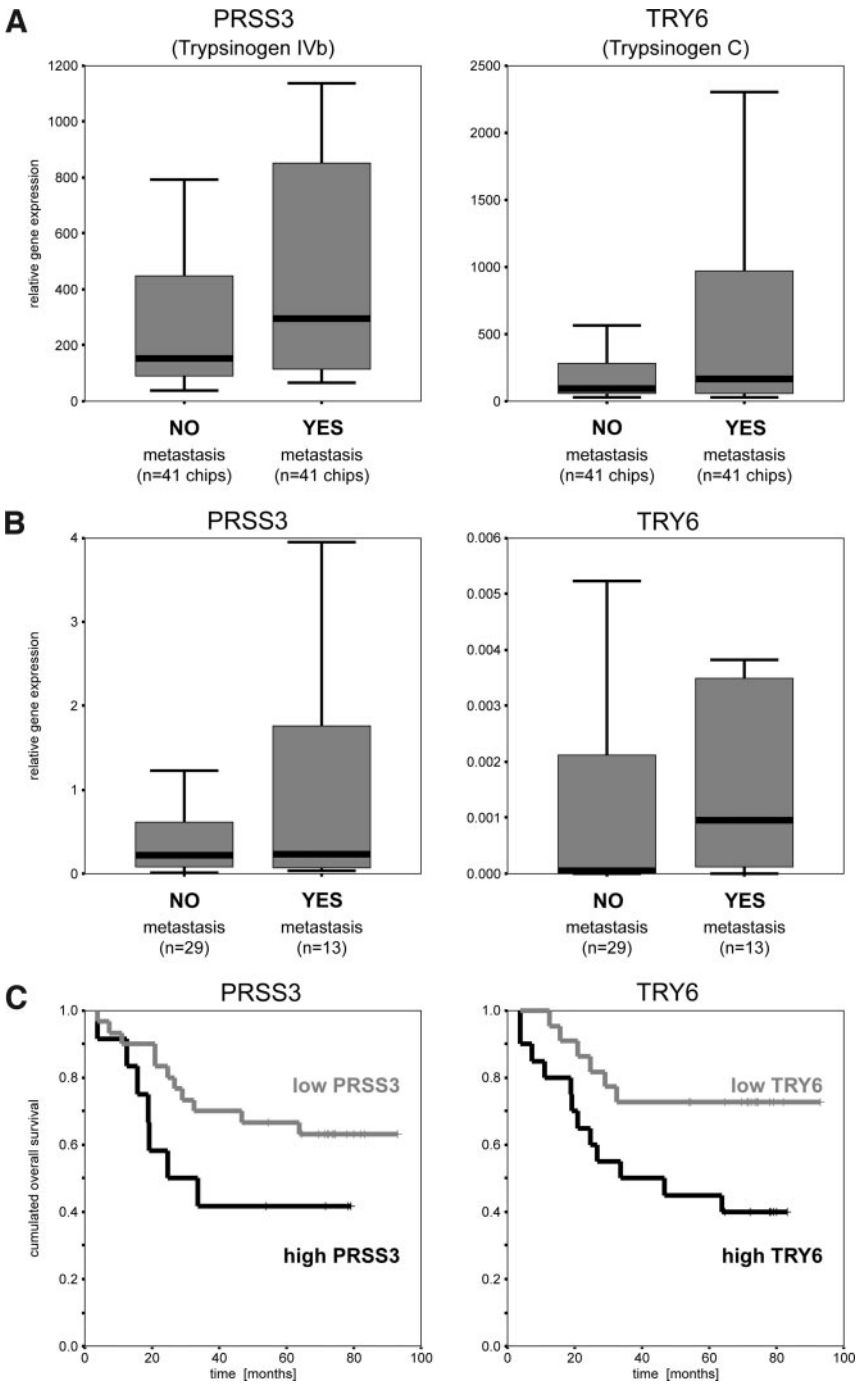

Fig. 3. Association of trypsinogens with metastasis and survival in NSCLC. *A*, the trypsin precursors trypsinogen IVb (*PRSS3*; $P = 0.046$) and trypsinogen C (*TRY6*) showed higher levels of expression in the metastasis-developing subgroup of NSCLC patients in the microarray study ($n = 82$ arrays). *B*, induction of PRSS3 and TRY6 was confirmed by real-time quantitative RT-PCR in an independent subset of patients ($n = 42$). *C*, survival analysis based on real-time RT-PCR data revealed inferior overall survival for patients expressing high levels of either PRSS3 ($P = 0.121$; nonsignificant) or TRY6 ($P = 0.031$).

(Fig. 4*C*, *control*) was used as loading control. The trypsinogen fusion proteins closely co-migrated with this nonspecific band, making it difficult to clearly distinguish them from each other (Fig. 4*C*).

Migration assays in Transwell plates ($n = 9$) revealed significantly increased migratory activity in cells overexpressing S100P ($P = 0.026$, Mann-Whitney *U* test) or S100A2 ($P = 0.001$). TRY6-overexpressing cells migrated less compared with control cells transfected with EGFP alone (Fig. 4*D*, *top panel*).

To study an experimental model simulating the *in vivo* situation of metastasis and invasion, we analyzed the migration through endothelial cell layers seeded into Transwell chambers ($n = 9$). When compared with EGFP-expressing controls, we detected a consistent and significant increase in spontaneous transendothelial migration of cell cultures stably overexpressing S100P, S100A2, PRSS3, and TRY6 ($P = 0.012$, $P = 0.001$, $P = 0.001$, and $P = 0.034$, respectively, Mann-Whitney *U* test; Fig. 4*D*, *bottom panel*).

Discussion

In this study, we identified differences in the gene expression patterns between primary NSCLC tumors that did or did not lead to distant metastasis. We compared microarrays hybridized in our laboratory with previously published data (7) and verified gene regulation with an alternative method in a third independent subset of patients. Therefore, our data reached a high level of validity since the important findings were confirmed in three independent patient groups.

The quality of our data is further corroborated by the frequent

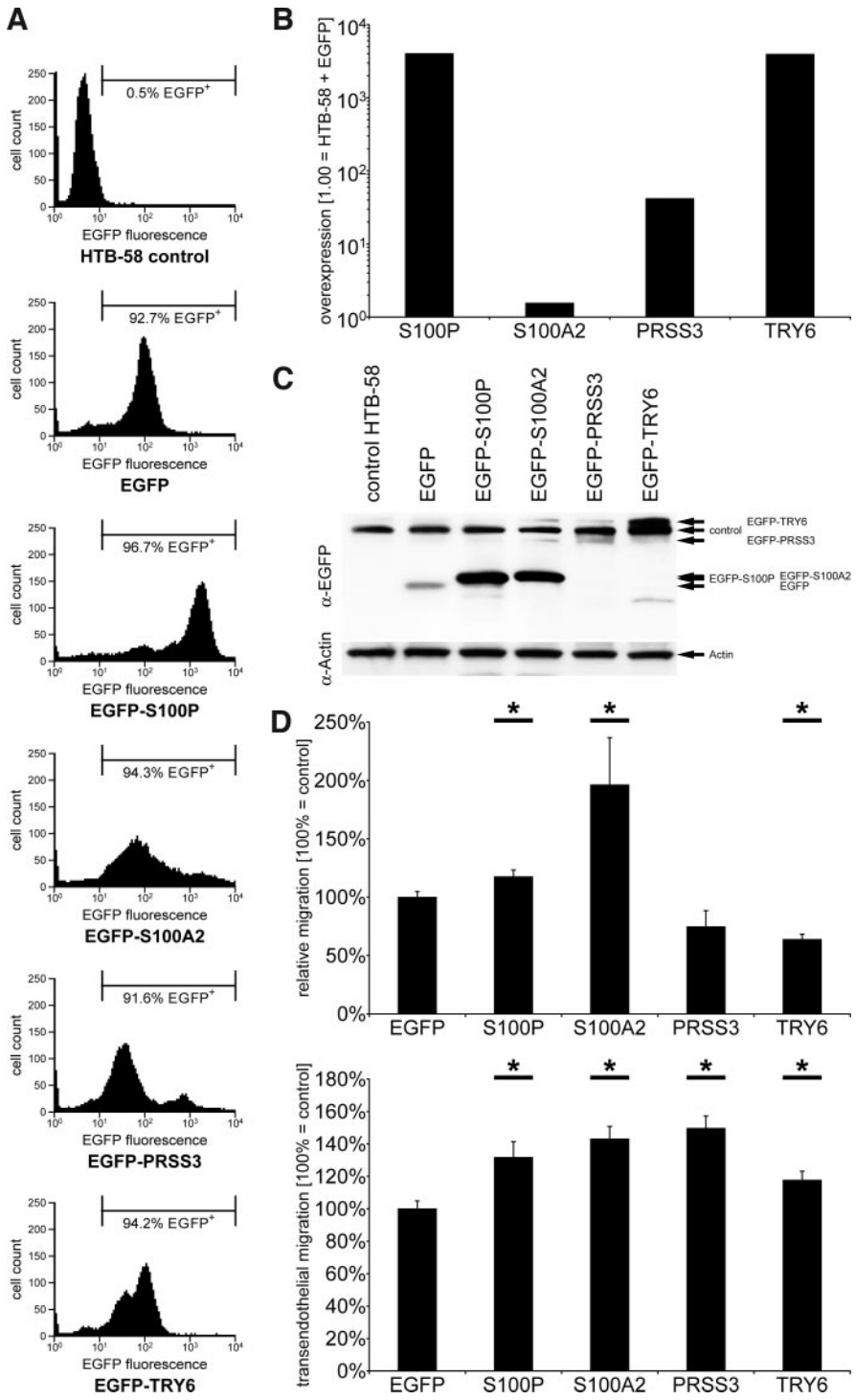

Fig. 4. S100 proteins and trypsinogens regulate migration/invasion. *A*−*C*, NSCLC cell cultures stably expressing S100 proteins and trypsinogens were established. Expression of EGFP fusion proteins of S100P, S100A2, PRSS3, and TRY6 was verified by FACS analysis (*A*), real-time quantitative RT-PCR (*B*), and Western blotting (*C*) with a nonspecific band and actin serving as loading controls. *D*, analysis of the migratory phenotype in Transwell chambers ($n = 9$) revealed significantly increased migration rates for cells expressing S100P ($P = 0.026$) and S100A2 ($P = 0.001$; *top panel*). To use an approach closer to the *in vivo* situation of metastasis and invasion, we performed transendothelial migration assays ($n = 9$). S100P, S100A2, PRSS3, and TRY6 overexpression led to enhanced migration through an endothelial cell layer compared with control cells. The differences were consistent and statistically significant ($P = 0.012$, $P = 0.001$, $P = 0.001$, and $P = 0.034$, respectively; *bottom panel*). Significant differences to control cells are marked with *asterisks*.

occurrence of genes in our study that are known to be involved in metastatic processes. The isolation of different members of one protein family in the list of regulated genes extends the probability of their importance *in vivo* in NSCLC metastasis. Our data are in accordance with most publications describing genes involved in metastasis because we found up-regulation of proteases that degrade extracellular matrix proteins, which is essential for migration and invasion. In addition, we detected proteins that were not yet linked to metastasis but to pathways assisting in this process. For example, we found up-regulation of inhibin A, a member of the tumor growth factor β family that is known to regulate the SMAD pathway (21) and to influence the expression of the known metastasis regulator plasminogen activator inhibitor-1 (22) or collagen, which was also down-regulated.

Notably, we also provide evidence for an involvement of gene families not yet assigned to NSCLC metastasis: S100 proteins and trypsinogens. Their induction in metastasizing tumors was verified in three independent patient groups using two different methods to ensure the general importance of the regulated genes for the underlying process.

S100 proteins build a family of 20 calcium-binding EF-hand proteins so far studied mainly in the immune system. They regulate

intracellular processes such as cell growth and motility, cell cycle, transcription, and differentiation. Individual members localize to specific cellular compartments and are able to relocate upon Ca^{2+} activation, transducing the Ca^{2+} signal by interacting with specific targets (23). The involvement of S100 proteins in migratory processes has not been recognized before in NSCLC but fits well into the general picture of calcium-dependent cell movement (16). S100A4 was the only family member described to participate in metastasis (18); therefore, our study for the first time provides evidence for an important role of S100A2 and S100P in metastasis and NSCLC survival.

Trypsins are serine proteases responsible for digestion in the duodenum and activated by autocatalytic cleavage from their trypsinogen precursors. They have been implicated in the progression of malignant diseases of the pancreas, the organ in which the trypsinogens are synthesized, and in the large bowel system (20). In the lung, trypsinogen has mainly been investigated with regard to inflammatory processes. Therefore, our results link trypsin activity for the first time to tumor progression and metastasis in organs unrelated to the digestive tract. Mechanistically, trypsins could assist in invasion by degrading proteins of the ECM.

In addition, stable overexpression of S100P, S100A2, PRSS3, and TRY6 in NSCLC cells revealed significantly enhanced transendothelial migration. Because only S100 proteins induced a migratory phenotype in the absence of endothelial cells, we assume that trypsinogens are not of major importance for migration in general; rather, they are important for specific processes such as invasion or evasion from blood vessels and, by this mechanism, are linked to metastasis.

Our study establishes a basis for the prediction of metastatic events at the time of diagnosis derived from gene expression analysis of the primary tumor. Our list of new markers for the development of metastasis, combined with published knowledge, will help to improve therapeutic decisions for early-stage NSCLC patients and create the future opportunity to apply adjuvant chemotherapy specifically to patients with the highest expected benefit from additional treatment.

Taken together, we have identified S100 proteins, trypsinogens, and more than 30 other genes associated with metastasis in early-stage NSCLC. High expression of several of these genes might be valuable for prediction of metastasis and survival. For the first time, this implicates a role for S100 proteins and trypsinogens in the metastatic process of early-stage NSCLC.

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