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Original Article

SIAH2 protein expression in breast cancer is inversely related with ER status and outcome to tamoxifen therapy

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Abstract: Our previous study demonstrated that high mRNA levels for *Seven in Absentia Homolog 2 (SIAH2)* correlated with high *Estrogen Receptor (ER)* mRNA levels and with longer progression-free survival (PFS) after first-line tamoxifen. Others showed high SIAH2 protein levels in ER-negative breast cancer associated with an unfavorable relapse-free survival. In the current study, we investigated SIAH2 protein expression to clarify the discrepancy between protein and mRNA findings and to determine its diagnostic value in breast cancer patients. Tissue microarrays (TMAs) containing core specimens of primary breast tumors were immunohistochemically stained for SIAH2 protein. The TMAs analyzed a cohort of 746 patients with primary breast cancer (PBC) and a cohort of 245 patients with ER-positive metastatic breast cancer (MBC) treated with first-line tamoxifen. SIAH2 staining was scored for intensity and proportion of positive tumor cells and evaluated for its relationship with metastasis-free survival (MFS) and PFS. Multivariate survival analyses included traditional prognostic or predictive factors, respectively. The PBC-cohort had 263 patients with high SIAH2 protein expression and decreased expression of ER protein and mRNA levels ($P = 0.005$ and $P = 0.003$, respectively). High SIAH2 levels correlated with significant unfavorable MFS in lymph node negative, ER-positive breast cancer patients. The MBC-cohort had 86 patients with increased SIAH2 protein expression. High SIAH2 expression was associated with an unfavorable PFS after first-line tamoxifen in multivariate analyses (HR = 1.45; 95% CI, 1.07-1.96; $P = 0.015$). In conclusion, SIAH2 protein expression is especially observed in ER-negative tumors. Its prognostic value in breast cancer does not add to current prognostic markers. The proportion of SIAH2-positive cells can be used as biomarker to predict tamoxifen treatment failure in MBC patients.

Keywords: Seven-in-absentia-homolog 2, breast cancer, endocrine therapy resistance, tissue microarray, immunohistochemistry

Introduction

Each year approximately 1.4 million women worldwide are diagnosed with breast cancer. Despite many new developments in the treatment of breast cancer, the overall survival (OS) of patients with metastatic breast cancer (MBC) remains poor [1]. Currently, Estrogen Receptor (ER)-positive breast cancer patients are treated with anti-hormonal agents such as tamoxifen and aromatase inhibitors. Although tamoxifen treatment results in tumor growth inhibition, in metastatic disease only half of the patients with ER-positive breast tumors responds to this drug. Moreover, those women who initially respond on tamoxifen, will eventually develop progressive disease due to acquired resistance. We revealed 81 genes differentially expressed between ER-positive breast cancer patients with progressive disease and patients who respond to tamoxifen [2]. *SIAH2* was one of the genes significantly related with response and progression-free survival (PFS) [3].

SIAH2 is an ubiquitin E3 ligase which ubiquitinates proteins for proteasomal-dependent degradation [3, 4] and has target proteins in the RAS and estrogen signaling pathway, DNA dam-

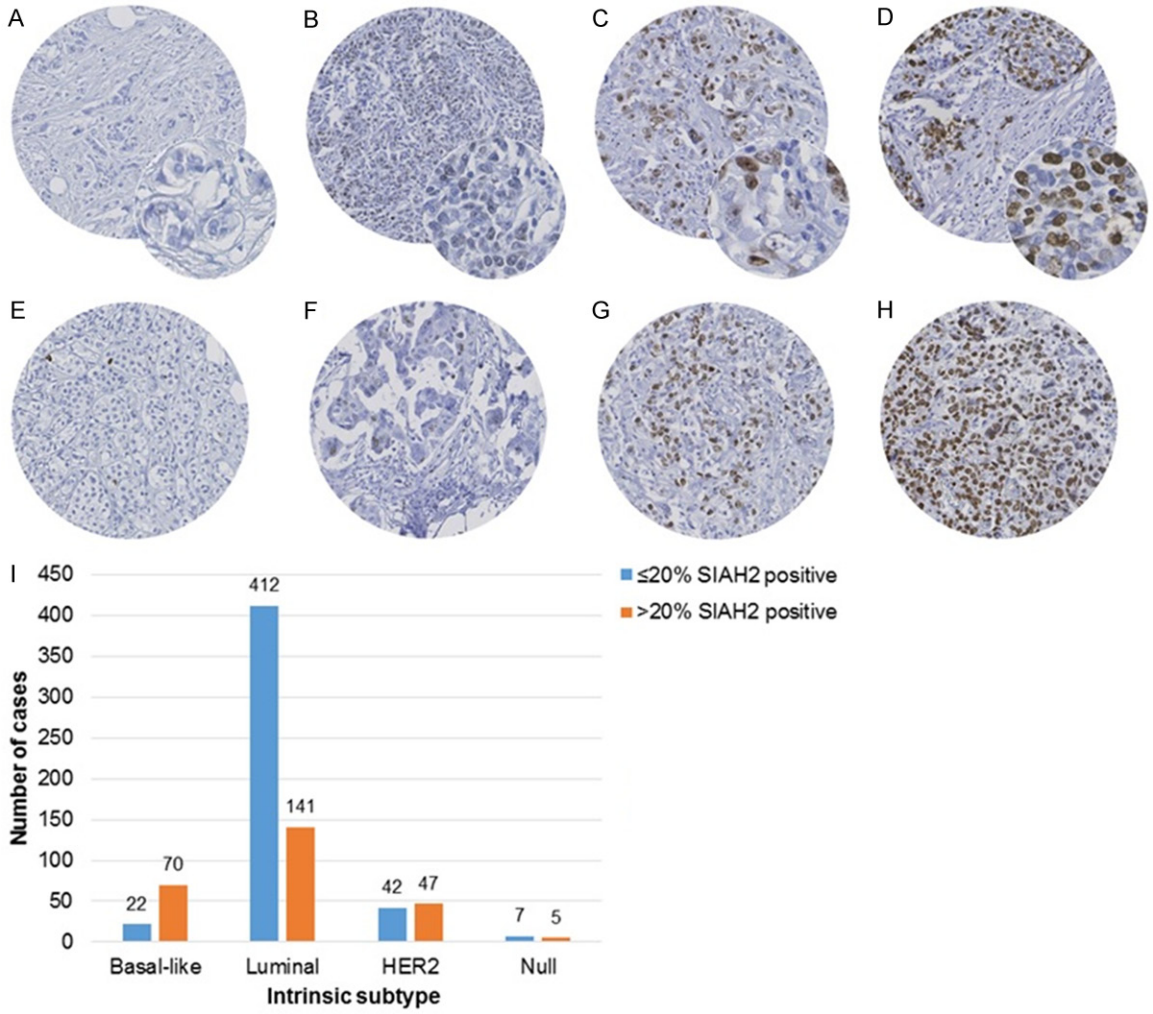

Figure 1. SIAH2 staining patterns and intrinsic subtypes. A to D exemplify the categories for staining intensity, whereas figures E to H those for proportion of SIAH2-positive tumor cells. A. Negative for SIAH2; B. Weak intensity; C. Moderate intensity; D. Strong intensity. E. Less than 5% of the tumor cells positive for SIAH2, strong staining. F. Moderate staining in 11-20% of the tumor cells. G. Moderate staining in 31-40% of the tumor cells. H. 100% of the tumor cells are strongly stained for SIAH2. I. Shows SIAH2 protein expression within the intrinsic subtypes. Analysis was performed on 746 tumors of the PBC-cohort. Protein expression of SIAH2 was dichotomized on the proportion of positive tumor cells, i.e. ≤ 20% SIAH2-positive versus > 20% SIAH-positive. The intrinsic subtypes were defined by ER, HER2/neu, EGFR and Cytokeratin 5 and classified according to Chan et al. [9] as luminal (positive for ER, negative for HER2/neu), HER2 (positive for HER2/neu), basal (positive for EGFR and/or Cytokeratin 5, negative for ER and HER2/neu) and null (negative for all). SIAH2-positive tumors are especially observed in the basal subtype (76%), in contrast to the luminal subtype (25%).

age response, cell growth and differentiation, angiogenesis and hypoxia [4-6]. Studies in breast cancer showed SIAH2 mainly in ER-positive tumors [3, 7]. In addition, estrogens upregulated SIAH2 on both mRNA and protein level by a rapid transcriptional response mediated by the ER [8].

Our previous study in primary breast tumors demonstrated a positive relationship between *SIAH2* mRNA and ER protein levels [3]. In contrast, Chan et al. found that SIAH2 protein levels were predominantly upregulated in ER-negative breast cancer [9]. They also observed increased SIAH2 protein expression during the transition of carcinoma *in situ* to invasive cancer and concluded that high SIAH2 protein expression is associated with an unfavorable survival. On the other hand, we observed increased *SIAH2* mRNA levels with a favorable

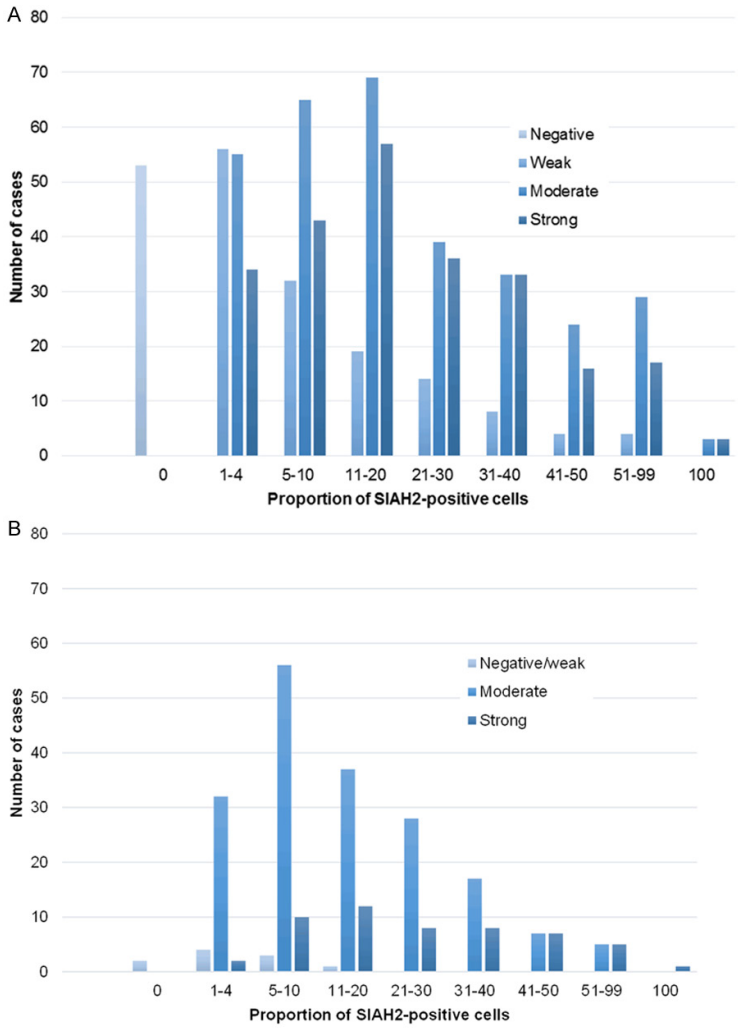

Figure 2. Overview of scoring categories for proportion of SIAH2-positive cells and staining intensity. The upper figure shows the distribution for SIAH2 protein staining intensity and amount of SIAH2-positive tumor cells in 746 tumors of patients from the PBC-cohort. The lower figure shows the staining intensity for SIAH2 and the amount of SIAH2-positive tumor cells in 245 patients from the MBC cohort.

disease outcome in patients with ER-positive primary breast cancer [3]. In addition, Confalonieri et al. showed a positive association between *SIAH2* mRNA and disease-free survival (DFS) [10]. As yet, it is unknown why SIAH2 protein and mRNA levels have opposed prognostic value in these heterogeneous tumor specimens.

In the present study, we investigated the relationship between SIAH2 and ER protein as well as mRNA expression levels to resolve the observed discrepancy in literature. We also examined the association of SIAH2 protein expression with prognosis and tamoxifen therapy response in ER-positive breast cancer patients.

Patients and methods

Ethics statement

This retrospective study was approved by the medical ethics committee of the Erasmus MC Rotterdam, the Netherlands (MEC 02.953). The study was carried out according the REMARK guidelines [11] and Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl).

Patients and tumor tissues

Formalin-fixed, paraffin-embedded (FFPE) primary breast tumor tissue samples were used from patients with primary operable breast cancer between 1985 and 2000. Two different patient series were included, i.e. patients with primary breast cancer (PBC-cohort) and patients with metastatic breast cancer treated with first-line tamoxifen monotherapy (MBC-cohort).

The PBC-cohort contained 817 patients. Tumors were included for analysis if histologic subtype, tumor differentiation grade according to the Bloom-Richardson score, ER, progesterone receptor (PgR), HER2/neu, epidermal growth factor receptor (EGFR) and cytokeratin 5 (CK5) status were available. After applying these criteria, tumor specimens of 746 patients were analyzed for SIAH2 protein expression.

Three hundred twenty six of these patients (43%) had breast-conserving surgery and 433 patients (57%) underwent modified mastectomy. Two hundred eighty two patients (38%) received adjuvant therapy, of which 108 patients were treated with hormonal therapy, 138 patients with chemotherapy and 36 patients with a combination of hormonal and chemotherapy. Six hundred twenty tumors (82%) were considered as ER-positive. Four hundred sixty four patients (62%) did not receive adjuvant systemic therapy. The median follow-up time of pa-

Table 1. Overview of SIAH2 protein expression quantity and intensity in patients from the primary breast cancer (PBC) and metastatic breast cancer (MBC) cohort

SIAH2 protein intensity	SIAH2 protein quantity in primary breast cancer cohort		SIAH2 protein quantity in metastatic breast cancer cohort	
	Low SIAH2 protein expression 0-20%	High SIAH2 protein expression > 20%	Low SIAH2 protein expression 0-20%	High SIAH2 protein expression > 20%
Negative	53	0		
Weak*	107	30	10	0
Moderate	189	128	125	57
Strong	134	105	24	29
Total	483	263	159	86

*Weak was defined as negative/weak in the metastatic breast cancer cohort. Pearson's Chi-squared test showed a positive correlation between SIAH2 protein intensity and quantity $P < 0.001$.

Table 2. Associations of SIAH2 protein levels with clinicopathological factors of 746 patients from the primary breast cancer (PBC) cohort

Characteristic	*N*	%	Low SIAH2 protein expression*	%	High SIAH2 protein expression**	%	*P*-value[α]
All patients	746	*100*	483	*65*	263	*35*	
Age (years)							
≤ 40	73	*10*	36	*7*	37	*14*	0.001[β]
41-55	309	*41*	192	*40*	117	*44*	
56-70	249	*33*	167	*35*	82	*31*	
> 70	115	*15*	88	*18*	27	*10*	
Menopausal status							
Premenopausal	347	*47*	205	*42*	142	*54*	0.003
Postmenopausal	399	*53*	278	*58*	121	*46*	
Tumor stage							
pT1	426	*57*	299	*62*	127	*48*	0.001
pT2/9	279	*37*	158	*33*	121	*46*	
pT3/4	41	*5*	26	*5*	15	*6*	
Lymph nodes involved							
0	410	*55*	259	*54*	151	*57*	0.132
1-3	217	*29*	152	*31*	65	*25*	
> 3	119	*16*	72	*15*	47	*18*	
Differentiation grade[#]							
1	142	*19*	135	*28*	7	*3*	< 0.001
2	334	*45*	248	*51*	86	*33*	
3	270	*36*	100	*21*	170	*65*	
Tumor histology							
IDC	653	*88*	426	*88*	227	*86*	0.055
ILC	28	*4*	22	*5*	6	*2*	
other	65	*9*	36	*7*	30	*11*	
ER status[†]							
Negative	136	*18*	45	*9*	91	*35*	< 0.001
Positive	610	*82*	438	*91*	172	*65*	
PgR status[†]							
Negative	275	*37*	129	*27*	146	*56*	< 0.001
Positive	471	*63*	354	*73*	117	*44*	

HER2/neu status†							
Negative	657	*88*	441	*91*	216	*82*	< 0.001
Positive	89	*12*	42	*9*	47	*18*	
EGFR expression†							
Negative	692	*93*	458	*95*	234	*89*	0.003
Positive	54	*7*	25	*5*	29	*11*	
CK5 expression†							
Negative	577	*77*	413	*86*	164	*62*	< 0.001
Positive	169	*23*	70	*14*	99	*38*	

Abbreviations: IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; ER, estrogen receptor; PgR, progesterone receptor; EGFR, epidermal growth factor receptor; CK5, cytokeratin 5. *SIAH2 negative defined as ≤ 20% cells positive for SIAH2 staining. **SIAH2 positive defined as > 20% cells positive for SIAH2 staining. [α]*P* for Pearson's Chi-squared test, [β]Mann-Whitney *U* test. [#]According to the Bloom-Richardson score. †As retrieved from TMA.

tients alive was 124 months (4 to 326 months) with a median metastasis-free survival (MFS) of 95 months (4 to 328 months). Disease recurrence occurred in 275 patients (36%).

Of the patients who developed metastatic breast cancer, 89 patients (32%) were treated with first-line tamoxifen monotherapy. These 89 patients were also included in the MBC-cohort. The MBC-cohort included 339 patients with metastatic disease and treated with first-line tamoxifen therapy. After applying above inclusion criteria, tumor specimens of 245 patients in this TAM cohort were eligible for further analyses.

Patient and tumor characteristics of this MBC-cohort have been previously reported by us [12]. Briefly, 33 of these 245 patients (13%) underwent breast-conserving surgery and 46 patients (19%) modified mastectomy. Eighty patients (33%) received adjuvant chemotherapy, the remaining 165 patients (67%) were hormone-naive. Median follow-up time of patients alive was 44 months (6 to 282 months). After developing metastatic disease, all 245 patients received first-line tamoxifen monotherapy. From the 162 patients (66%) who benefit from this treatment, 8 patients (5%) had a complete response, 43 patients (27%) had a partial response and 111 patients (69%) had stable disease for more than six months. Therapy failure was observed in 83 patients (34%) of whom 60 patients (72%) showed progressive disease and 23 patients (28%) had stable disease for six months or less.

Tissue microarray immunohistological staining and evaluation

Preparation of tissue microarrays (TMAs) of FFPE primary breast tumor specimens and immunohistochemical stainings were performed as described previously [12]. For immunohistochemistry the primary antibody against SIAH2 (monoclonal (1:80), Novus Biologicals, Littleton, CO, USA) was incubated for one hour at room temperature. Stainings were scored by at least two observers independently (AT, KvdW, ER). The scoring was performed similar to the method described previously [13] and determined staining intensity and proportion of tumor cells with SIAH2 expression. The proportion of cells with SIAH2 expression was divided into nine groups (0 = negative for SIAH2 staining, 1 = 1-4% SIAH2 positive cells, 2 = 5-10%, 3 = 11-20%, 4 = 21-30%, 5 = 31-40%, 6 = 41-50%, 7 = 51-99%, 8 = 100%). Staining intensity was split into four categories (0 = negative, 1 = weak, 2 = moderate, 3 = strong). The scoring method by Chan et al. was also used to evaluate our SIAH2 staining [9]. This method combines both intensity (same categories as above) and proportion tumor cells stained for SIAH2 (0 = no cells staining positive, 1 ≤ 10%, 2 = 10-50%, 3 ≥ 50%). The scores for intensity and percentage were added up to a maximum of 6. A cut-off of > 2 was considered as SIAH2 positive.

Next to the protein expression levels also mRNA levels of *SIAH2* and *ER* were available for 114 patients from the PBC-cohort [3].

Data analysis and statistics

Statistical analysis were performed with STATA statistical package, release 13.0 (STATA Corp., College Station, TX), similarly as described previously [12]. Log-rank tests for trends and when appropriate the Pearson's Chi-squared and Mann-Whitney were used to investigate the association between SIAH2 protein expression

Table 3. Associations of SIAH2 protein levels with clincopathological factors of 245 patients from the MBC-cohort

Characteristic	*N*	%*	Low SIAH2 protein expression**	%	High SIAH2 protein expression***	%	*P*-value[α]
All patients	245	*100*	159	*65*	86	*35*	
Age (years)							
≤ 40	17	*7*	8	*5*	9	*10*	0.003[β]
41-55	78	*32*	44	*28*	34	*40*	
56-70	88	*36*	60	*38*	28	*33*	
> 70	62	*25*	47	*30*	15	*17*	
Menopausal status							
Premenopausal	66	*27*	33	*21*	33	*38*	0.003
Postmenopausal	179	*73*	126	*79*	53	*62*	
Adjuvant chemotherapy							
No	148	*60*	97	*61*	51	*59*	0.790
Yes	80	*33*	50	*31*	30	*35*	
Lymph nodes involved							
0	86	*35*	49	*31*	37	*43*	0.107
1-3	71	*29*	52	*33*	19	*22*	
> 3	83	*34*	53	*33*	30	*35*	
Differentiation grade[#]							
1	37	*15*	33	*21*	4	*5*	< 0.001
2	131	*53*	92	*58*	39	*45*	
3	77	*31*	34	*21*	43	*50*	
Tumor histology							
IDC	210	*86*	134	*84*	76	*88*	0.599
ILC	20	*8*	15	*9*	5	*6*	
Other	15	*6*	10	*6*	5	*6*	
PgR status[†]							
Negative	62	*25*	40	*25*	22	*26*	0.942
Positive	183	*75*	119	*75*	64	*74*	
HER2/neu status[†]							
Negative	197	*80*	140	*88*	57	*66*	< 0.001
Positive	48	*20*	19	*12*	29	*34*	
Dominant site of relapse							
Viscera	92	*38*	62	*39*	30	*35*	0.005
Bone	124	*51*	86	*54*	38	*44*	
Soft tissue	29	*12*	11	*7*	18	*21*	
Disease-free survival (years)							
≤ 1	36	*15*	25	*16*	11	*13*	0.611
1-3	118	*48*	73	*46*	45	*52*	
> 3	91	*37*	61	*38*	30	*35*	

Abbreviations: IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; PgR, progesterone receptor. *Due to missing information numbers do not always add up to 100%. **SIAH2 negative defined as ≤ 20% cells positive for SIAH2 staining. ***SIAH2 positive defined as > 20% cells positive for SIAH2 staining. [α]*P* for Pearson's Chi-squared test, [β]Mann-Whitney *U* test. [#]According to the Bloom-Richardson score. [†]As retrieved from TMA.

and clinicopathological factors. Boxplots were generated to illustrate correlations between protein and mRNA levels and scatterplots were used to visualize relationships between mRNA levels. Spearman rank correlation tests were applied to evaluate the relationships between protein and mRNA levels for the different molecular factors. Hazard ratios (HR) with 95%

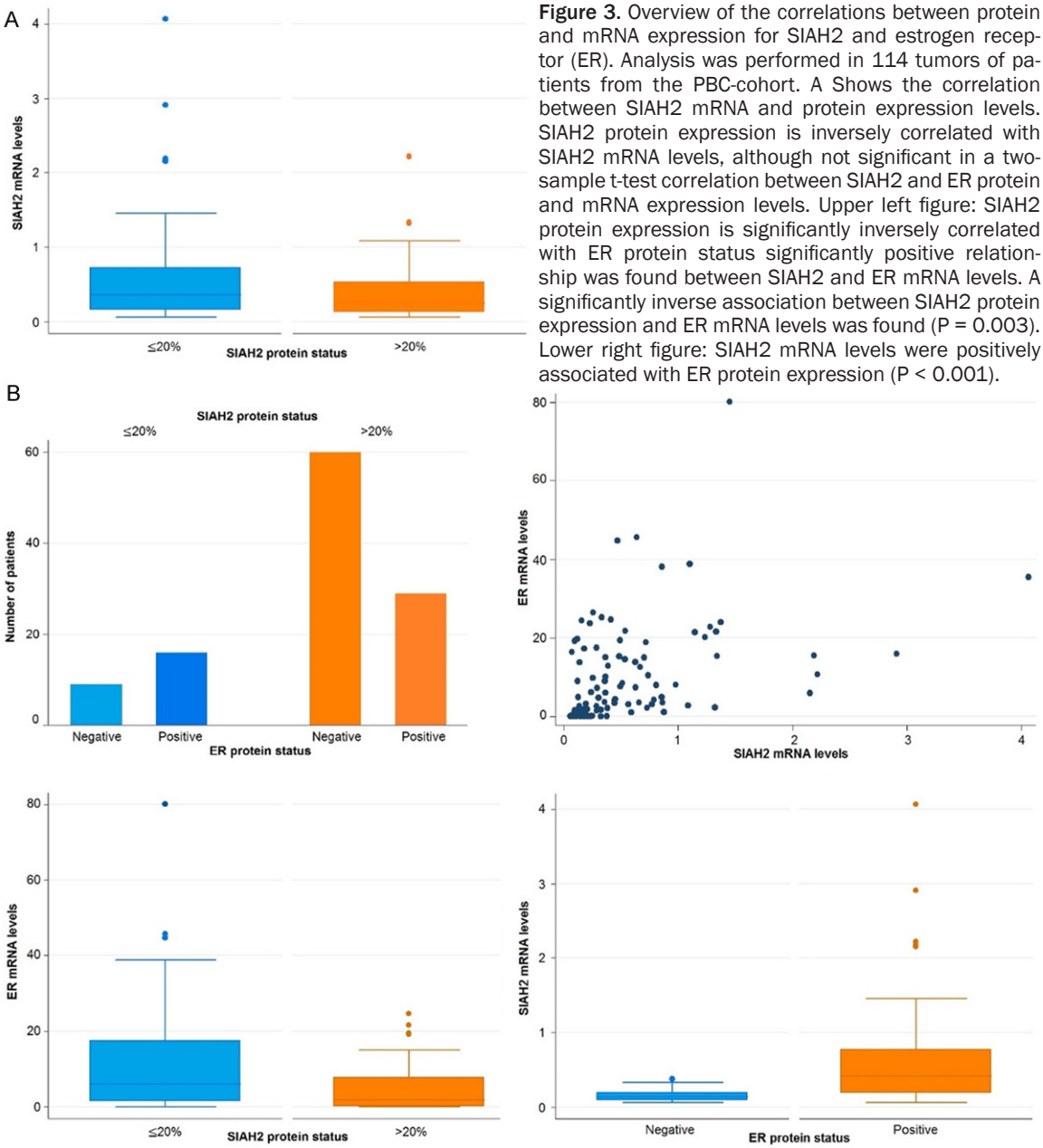

Figure 3. Overview of the correlations between protein and mRNA expression for SIAH2 and estrogen receptor (ER). Analysis was performed in 114 tumors of patients from the PBC-cohort. A Shows the correlation between SIAH2 mRNA and protein expression levels. SIAH2 protein expression is inversely correlated with SIAH2 mRNA levels, although not significant in a two-sample t-test correlation between SIAH2 and ER protein and mRNA expression levels. Upper left figure: SIAH2 protein expression is significantly inversely correlated with ER protein status significantly positive relationship was found between SIAH2 and ER mRNA levels. A significantly inverse association between SIAH2 protein expression and ER mRNA levels was found (P = 0.003). Lower right figure: SIAH2 mRNA levels were positively associated with ER protein expression (P < 0.001).

CI were computed by the Cox proportional hazard model in order to analyze the association of SIAH2 protein expression with MFS for the PBC-cohort and with PFS after first-line therapy with tamoxifen for the MBC-cohort. The endpoints MFS and PFS were defined as described previously [14]. Significant findings in univariate analysis for SIAH2 expression were compared in multivariate analysis with our base models of traditional clinicopathological predictors to test for its independent prognostic and predictive value. Survival curves were generated by using the Kaplan-Meier method. The log-rank test was used to test for differences between survival curves. The *P*-values were two sided and significant. P < 0.05 was considered as statistically significant.

Results

SIAH2 protein expression

Different staining patterns for SIAH2 were observed, as exemplified in **Figure 1A-H**. Its expression was predominantly detected in the nucleus as described previously [15-18]. The proportion of cells expressing SIAH2 protein

Table 4. Cox univariate and multivariate analysis for metastasis-free survival of SIAH2 protein expression levels. Analysis was performed in all 746 patients from the PBC-cohort

			Univariate analysis			Multivariate analysis		
Factor of base model	*N*	%	HR	95% CI	*P*-value	HR	95% CI	*P*-value
All patients	746	*100*						
Age (years)								
≤ 40	73	10	1.00			1.00		
41-55	309	*41*	0.66	0.46 to 0.95	0.027	0.80	0.55 to 1.16	0.243
56-70	249	*33*	0.58	0.40 to 0.86	0.006	0.91	0.48 to 1.71	0.767
> 70	115	*15*	0.57	0.36 to 0.92	0.023	0.86	0.42 to 1.75	0.673
Menopausal status								
Premenopausal	347	47	1.00			1.00		
Postmenopausal	399	*53*	0.79	0.62 to 1.01	0.057	0.82	0.49 to 1.37	0.452
Tumor stage								
pT1	426	*57*	1.00			1.00		
pT2/9	279	*37*	1.85	1.44 to 2.37	< 0.001	1.50	1.15 to 1.94	0.002
pT3/4	41	*5*	2.75	1.76 to 4.30	< 0.001	1.89	1.19 to 3.00	0.007
Lymph nodes involved								
0	410	*55*	1.00			1.00		
1-3	217	*29*	1.61	1.22 to 2.13	0.001	1.49	1.12 to 1.97	0.006
> 3	119	*16*	2.92	2.16 to 3.93	< 0.001	1.76	1.76 to 3.31	< 0.001
Differentiation grade								
1	85	*25*	1.00			1.00		
2	164	*49*	2.44	1.30 to 4.59	0.006	2.30	1.21 to 4.34	0.011
3	87	*26*	3.24	1.67 to 6.30	0.001	3.14	1.58 to 6.27	0.001
ER status†								
Negative	136	*18*	1.00			1.00		
Positive	610	*82*	0.79	0.59 to 1.07	0.124	0.95	0.64 to 1.41	0.800
PgR status†								
Negative	275	37	1.00			1.00		
Positive	471	*63*	0.91	0.71 to 1.16	0.429	1.18	0.85 to 1.64	0.316
HER2 status†								
Negative	657	*88*	1.00			1.00		
Positive	89	*12*	1.39	0.99 to 1.95	0.056	1.25	0.88 to 1.78	0.208
SIAH2 protein expression						Added to the base model		
Low (≤ 20% positive cells)	483	*65*	1.00			1.00		
High (> 20% positive cells)	263	*35*	1.41	1.11 to 1.80	0.005	1.09	0.82 to 1.43	0.565

Abbreviations: HR, hazard ratio; ER, estrogen receptor; PgR, progesterone receptor. †As retrieved from TMA.

and the staining intensity were evaluated separately for all scoring categories in both cohorts of patients (**Figure 2**). Logrank tests for trend showed that only the categories for proportion but not for intensity were related with MFS in the PBC-cohort (proportion $P = 0.013$; intensity $P = 0.54$) and with PFS in the MBC-cohort (proportion $P = 0.005$; intensity $P = 0.67$). The method of Chan et al. [9], which combines proportion and intensity resulting in six scores, demonstrated a relationship with PFS ($P = 0.049$) but not with MFS ($P = 0.60$). Based on above findings, further analyses of SIAH2 protein expression as dichotomized variable were based on the proportion of SIAH2-positive cells (**Table 1**). Specimens with ≤ 20% positive cells were considered to express low SIAH2 protein levels, whereas tumors with > 20% positive cells were defined to have high SIAH2 protein expression. This resulted in 263 tumors (35%)

Table 5. Cox univariate and multivariate analysis for progression-free survival of SIAH2 protein expression levels. Analysis was performed in 610 patients with ER-positive breast cancer from the PBC-cohort

			Univariate analysis			Multivariate analysis		
Factor of base model	*N*	%	HR	95% CI	*P*-value	HR	95% CI	*P*-value
All patients	610	*100*						
Age (years)								
≤ 40	53	9	1.00			1.00		
41-55	250	*41*	0.64	0.42 to 0.97	0.034	0.74	0.48 to 1.13	0.165
56-70	208	*34*	0.54	0.35 to 0.83	0.006	0.64	0.31 to 1.31	0.221
> 70	99	*16*	0.57	0.33 to 0.97	0.037	0.67	0.30 to 1.49	0.330
Menopausal status								
Premenopausal	280	*46*	1.00			1.00		
Postmenopausal	330	*54*	0.83	0.63 to 1.08	0.159	1.04	0.58 to 1.86	0.898
Tumor stage								
pT1	361	*59*	1.00			1.00		
pT2/9	221	*36*	1.88	1.43 to 2.48	< 0.001	1.51	1.13 to 2.01	0.005
pT3/4	28	*5*	2.50	1.45 to 4.29	0.001	1.81	1.04 to 3.18	0.037
Lymph nodes involved								
0	336	*55*	1.00			1.00		
1-3	180	30	1.52	1.12 to 2.08	0.008	1.37	1.00 to 1.88	0.048
> 3	94	*15*	2.77	1.98 to 3.87	< 0.001	2.30	1.61 to 3.29	< 0.001
Differentiation grade								
1	138	*23*	1.00			1.00		
2	307	*50*	2.04	1.36 to 3.07	0.001	1.70	1.12 to 2.57	0.013
3	165	*27*	2.86	1.76 to 4.39	< 0.001	2.47	1.58 to 3.86	< 0.001
PgR status†								
Negative	146	*24*	1.00			1.00		
Positive	464	*76*	0.94	0.69 to 1.28	0.690	1.11	0.79 to 1.54	0.552
HER2 status†								
Negative	553	*91*	1.00			1.00		
Positive	57	*9*	1.53	1.02 to 2.31	0.041	1.25	0.81 to 1.92	0.318
SIAH2 protein expression						Added to the base model		
Low (≤ 20% positive cells)	438	*72*	1.00			1.00		
High (> 20% positive cells)	172	*28*	1.42	1.07 to 1.89	0.014	1.00	0.72 to 1.37	0.977

Abbreviations: HR, hazard ratio; PgR, progesterone receptor. †As retrieved from TMA.

and 86 tumors (35%) with high SIAH2 protein expression in the PBC- and MBC-cohort, respectively.

Association of SIAH2 protein expression with clinicopathological characteristics

SIAH2 protein expression in the PBC-cohort was significantly associated with all studied clinicopathological factors, except for the number of involved lymph nodes and tumor histology (**Table 2**). A positive correlation was detected between high SIAH2 protein expression and basal-like, HER2 and null intrinsic subtypes ($P < 0.001$), as shown in **Figure 1I**. In the ER-positive MBC-cohort, SIAH2 protein expression correlated with age, menopausal status, tumor differentiation grade, HER2/neu receptor expression and soft tissue metastases (**Table 3**).

Correlations between SIAH2 and ER on protein and mRNA levels in the PBC-cohort

We previously reported increased *SIAH2* mRNA expression in ER-positive compared to ER-negative tumors [3], whereas in this study higher SIAH2 protein levels in ER negative tumors were detected (**Table 2**; $P < 0.001$). To evaluate

Table 6. Cox univariate and multivariate analysis for metastasis-free survival of SIAH2 protein expression levels. Analysis was performed in 336 patients with lymph node-negative ER-positive breast cancer from the PBC-cohort

			Univariate analysis			Multivariate analysis		
Factor of base model	*N*	%	HR	95% CI	*P*-value	HR	95% CI	*P*-value
All patients	336	*100*						
Age (years)								
≤ 40	25	*7*	1.00			1.00		
41-55	133	*40*	0.53	0.29 to 0.99	0.048	0.63	0.33 to 1.20	0.162
56-70	120	*36*	0.34	0.18 to 0.69	0.002	0.25	0.09 to 0.74	0.012
> 70	58	*17*	0.54	0.26 to 1.17	0.119	0.38	0.12 to 1.20	0.101
Menopausal status								
Premenopausal	147	*44*	1.00			1.00		
Postmenopausal	189	*56*	0.80	0.53 to 1.20	0.282	1.75	0.76 to 4.02	0.184
Tumor stage								
pT1	235	*70*	1.00			1.00		
pT2/9	93	*28*	1.58	1.02 to 2.46	0.041	1.34	0.85 to 2.11	0.211
pT3/4	8	*2*	4.39	1.88 to 10.2	0.001	3.18	1.29 to 7.82	0.012
Differentiation grade								
1	85	*25*	1.00			1.00		
2	164	*49*	2.44	1.30 to 4.59	0.006	2.30	1.21 to 4.34	0.011
3	87	*26*	3.24	1.67 to 6.30	0.001	3.14	1.58 to 6.27	0.001
PgR status†								
Negative	78	*23*	1.00			1.00		
Positive	258	*77*	1.03	0.63 to 1.68	0.916	1.11	0.66 to 1.90	0.687
HER2 status†								
Negative	301	*90*	1.00			1.00		
Positive	35	*10*	1.69	0.96 to 2.99	0.070	1.21	0.65 to 2.27	0.553
SIAH2 protein expression						Added to the base model		
Low (≤ 20% positive cells)	241	*72*	1.00			1.00		
High (> 20% positive cells)	95	*28*	1.76	1.15 to 2.67	0.009	1.10	0.66 to 1.83	0.707

Abbreviations: HR, hazard ratio; PgR, progesterone receptor. †As retrieved from TMA.

this discrepancy, we investigated mRNA levels with protein levels for both genes, which were available for 114 patients. For *SIAH2* alone, mRNA levels had an inverse relation with protein expression (**Figure 3**; $P = 0.089$). Combined, SIAH2 protein levels were inversely associated with ER protein and mRNA levels (**Figure 3B**; $P = 0.005$ and $P = 0.003$, respectively), whereas *SIAH2* mRNA levels showed a positive correlation with mRNA and protein levels of ER (**Figure 3B**; $P < 0.001$).

Association of SIAH2 protein expression with MFS

High SIAH2 protein expression in the PBC-cohort was associated with an unfavorable MFS (HR = 1.41, CI 1.11-1.80, $P = 0.005$; **Table 4**). This association between SIAH2 protein expression and MFS continued to be significant in ER-positive tumors of 610 patients (HR = 1.42, CI 1.07-1.89, $P = 0.014$; **Table 5**) and within the prognostic subset of 336 lymph node-negative adjuvant systemic therapy naïve patients (HR = 1.76, CI, 1.15-2.67; $P = 0.009$; **Table 6**). The prognostic value of SIAH2 protein expression was visualized with a Kaplan-Meier curve in **Figure 4A**. SIAH2 protein expression and MFS were not significantly related in multivariate analysis, when corrected for traditional prognostic factors such as age, menopausal status, tumor stage, differentiation grade, PgR and HER2/neu status. Tumors with high SIAH2 protein expression were only related with OS for the whole set (HR = 1.29, CI 1.01-1.65; $P = 0.039$) but not for the subsets (HR = 1.26, $P =$

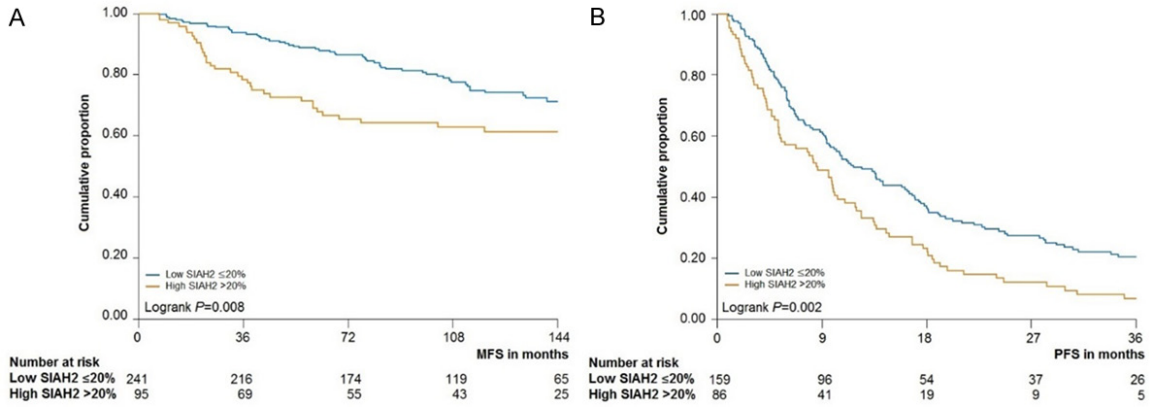

Figure 4. Kaplan-meier curves of MFS and PFS as a function of SIAH2 protein. A. Relationship between SIAH2 protein expression and metastasis-free survival in PBC cohort. Analysis was performed in 336 patients with lymph node negative, ER-positive breast cancer. SIAH2 protein expression was divided in tumors with low (≤ 20%, blue line) and high expression (> 20%, orange line). B. Relationship between SIAH2 protein expression and PFS analyzed in 245 MBC patients treated with first-line tamoxifen. SIAH2 protein expression was divided in tumors with low (≤ 20%) and high expression (> 20%).

0.19 and HR = 1.36, P = 0.160 for the ER-positive and prognostic subset, respectively).

Association of SIAH2 protein expression with PFS after first-line tamoxifen monotherapy

The MBC-cohort showed a significant relationship between SIAH2 protein expression and PFS (HR = 1.55, CI 1.18-2.04; P = 0.002; **Table 7** and **Figure 4B**) but not with OS after start of first-line therapy (HR = 1.21, CI 0.90-1.63, P = 0.202). When added to the multivariate base model including the traditional predictive factors age, menopausal status, adjuvant therapy, dominant site of relapse, DFS, PgR, and HER2/neu status, tumors with high SIAH2 protein expression associated with a poor PFS (HR = 1.45, CI 1.07-1.96; P = 0.015).

Discussion

In the current study, we investigated the relationship between SIAH2 and ER status and the prognostic and predictive value of SIAH2 in breast cancer patients. We show that SIAH2 protein expression is inversely related with ER protein expression. In contrast, we found a positive correlation between *SIAH2* mRNA levels and ER protein expression. Furthermore, we demonstrated that high SIAH2 protein expression is associated with an unfavorable MFS in PBC patients and PFS in MBC patients treated with first-line tamoxifen, respectively.

The observed inverse correlation between SIAH2 and ER protein expression has been described earlier in basal subtype of breast cancer by Chan et al. [9]. We now confirmed their findings in a larger subset of breast cancer patients and demonstrate high SIAH2 protein expression in especially ER-negative tumors. However, our previous study reported high *SIAH2* mRNA levels in tumors with high *ER* mRNA levels [3]. Exploratory analyses in a subset of patients for which mRNA as well as protein expression data were available confirmed the positive correlation at mRNA but the inverse relation at protein level between SIAH2 and ER. Discordance between protein and mRNA expression levels have also been observed for other genes [19]. Moreover, the proteo-genomic characterization of colon cancer revealed that mRNA transcript abundance is not automatically translated into protein abundance; only 32% of the genes had a significant positive mRNA-protein correlation [20].

Discrepancies between protein and mRNA levels have been explained by different mechanisms [21]. In our subset of tumors with high *SIAH2* mRNA levels, we observed a trend towards decreased SIAH2 protein levels. This finding might be explained by auto-ubiquitination, since most RING-finger domain E3 ubiquitin ligases, like SIAH2, have the capacity to ubiquitinate themselves, resulting in their own degradation and its limited availability to ubiquitinate target substrates [22]. Another possible explanation for the difference in SIAH2 protein and mRNA levels is that *SIAH2* mRNA sequences are complementary bound by microRNAs (miRNAs). These miRNAs are involved in the post-transcriptional (down) regulation of

Table 7. Cox univariate and multivariate analysis for progression-free survival of SIAH2 protein expression levels. Analysis was performed in 245 ER-positive tumors from patients with MBC treated with first-line tamoxifen monotherapy

			Univariate analysis			Multivariate analysis		
Factor of base model	*N*	%*	HR	95% CI	*P*-value	HR	95% CI	*P*-value
All patients	245	*100*						
Age (years)								
≤ 40	17	*7*	1.00			1.00		
41-55	78	*32*	1.21	0.71 to 2.08	0.487	1.19	0.68 to 2.07	0.545
56-70	88	*36*	0.69	0.40 to 1.18	0.173	0.53	0.26 to 1.07	0.076
> 70	63	*26*	0.56	0.32 to 0.98	0.044	0.44	0.21 to 0.95	0.038
Menopausal status								
Premenopausal	66	*27*	1.00			1.00		
Postmenopausal	180	*73*	0.64	0.48 to 0.86	0.003	1.15	0.73 to 1.80	0.544
Adjuvant chemotherapy								
No	149	*61*	1.00			1.00		
Yes	80	33	1.49	1.12 to 1.99	0.006	0.99	0.70 to 1.40	0.950
Dominant site of relapse								
Viscera	93	*38*	1.00			1.00		
Bone	124	*51*	0.81	0.61 to 1.07	0.144	0.70	0.52 to 0.95	0.021
Soft tissue	29	*12*	0.49	0.30 to 0.78	0.003	0.45	0.28 to 0.73	0.001
Disease-free survival (years)								
≤ 1	36	*15*	1.00			1.00		
1-3	118	*48*	0.78	0.53 to 1.15	0.218	0.69	0.41 to 1.16	0.157
> 3	92	*38*	0.70	0.47 to 1.05	0.083	0.63	0.37 to 1.10	0.103
PgR status†								
Negative	62	*25*	1.00			1.00		
Positive	184	*75*	0.87	0.64 to 1.17	0.357	0.79	0.58 to 1.09	0.152
HER2/neu status†								
Negative	198	*81*	1.00			1.00		
Positive	48	*20*	1.15	0.82 to 1.60	0.414	0.98	0.69 to 1.39	0.901
SIAH2 protein expression						Added to the base model		
Low (≤ 20% positive cells)	86	*35*	1.00			1.00		
High (> 20% positive cells)	159	*65*	1.55	1.18 to 2.04	0.002	1.45	1.07 to 1.96	0.015

Abbreviations: HR, hazard ratio; PgR, progesterone receptor. *Due to missing information numbers do not always add up to 100%. †As retrieved from TMA.

gene expression and affects both translation and stability of target mRNAs. Several miRNAs have been identified to be differentially expressed between the molecular subtypes of breast cancer [30]. Until now, only miR-146b-5p has been functionally shown to target and down-regulate SIAH2 mRNA *in vitro* and *in vivo* [23, 24] and was detected in breast cancer [25, 26].

In the present study, we demonstrate that high SIAH2 protein expression in ER-positive primary tumors is associated with unfavorable MFS in PBC patients and with PFS after first-line tamoxifen monotherapy in MBC patients. The diagnostic value of SIAH2 has previously been investigated in several other studies. Chan et al. also showed in 246 tumors, including basal and luminal subtypes, that high levels of SIAH2 protein expression associated with an unfavorable survival [9]. At the mRNA level, our previous study showed that high *SIAH2* levels were related with prolonged PFS [3] and others associated high *SIAH2* levels with a longer DFS, MFS and OS [3, 10]. These differences in prognostic and predictive value of SIAH2 protein versus transcript in the various studies can be explained by our observed inverse relation

between SIAH2 protein and mRNA expression levels.

Our results suggest that tamoxifen resistance in tumor specimens can be defined by low mRNA levels and high protein expression for SIAH2. A role of SIAH2 in endocrine therapy resistance might be anticipated because SIAH2 expression is upregulated by estrogens via ER [8]. Since high SIAH2 protein levels are correlated with low ER expression in this study, the question arises whether SIAH2 controls in a feedback loop the ER protein levels. The resistance to tamoxifen might then be explained by a lack of target due to SIAH2 mediated degradation of ER. Further studies are needed to investigate this hypothesis and to determine the role of SIAH2 in endocrine therapy resistance.

Compounds that target SIAH2 protein might help to overcome endocrine therapy resistance. Next to general proteasome inhibitors that might be applicable, only one SIAH2 specific protein inhibitor has been identified and tested *in vivo*. The compound menadione (vitamin K3) attenuates SIAH2 auto-ubiquitination which resulted in inhibition of tumor growth in mice with xenografted human melanomas [27]. Reducing SIAH2 activity by menadione in breast cancer might repress ER-signaling due to accumulation of SIAH2 targets N-CoR and HDAC3 [28, 29], two ER transcriptional repressor proteins. On the other hand, our *in vitro* results showed that SIAH2 silencing was associated with decreased sensitivity to the pure antiestrogen ICI164,384 [3]. The effect of SIAH2 inhibition could also be realized by reducing hypoxia-inducible factor 1α, which can enable tumor growth and metastasis by inducing angiogenesis [30].

To summarize, we have demonstrated an inverse relationship between SIAH2 mRNA and protein expression levels. High SIAH2 protein expression is especially observed in ER-negative breast cancer. In ER-positive breast cancer, high levels of SIAH2 protein associate with unfavorable outcome in PBC and treatment outcome in MBC. Together with the mRNA findings in our previous study, we are the first to report a relationship between SIAH2 protein expression and outcome to tamoxifen in MBC. Assessment of SIAH2 mRNA and protein levels could allow a better selection of patients for tamoxifen when our findings are confirmed.

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Disclosure of conflict of interest

None.

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