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Downregulation of SIAH2, an ubiquitin E3 ligase, is associated with resistance to endocrine therapy in breast cancer

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Abstract *Purpose* In our microarray analysis we observed that Seven-in-Absentia Homolog 2 (SIAH2) levels were low in estrogen receptor (ER) positive breast tumors of patients resistant to first-line tamoxifen therapy. The aim of this study was to evaluate SIAH2 for its (a) predictive/prognostic value, and (b) functional role in endocrine therapy resistance. *Patients and methods* SIAH2 expression was measured with quantitative Real-Time-PCR (qRT-PCR) in 1205 primary breast tumor specimens and related to disease outcome. The functional role of SIAH2 was determined in human breast cancer cell lines ZR-75-1, ZR/HERc, and MCF7. Cell lines were treated with estrogen (E2), anti-estrogen ICI164.384 or epidermal growth factor (EGF). Moreover, MCF7 was treated with ICI164.384 after silencing SIAH2 expression. *Results* SIAH2 was not prognostic in 603 lymph node negative patients who had not received adjuvant systemic therapy. In multivariate analysis of ER-positive tumors of 235 patients with recurrent disease, SIAH2 as continuous

variable, significantly predicted first-line tamoxifen treatment failure (OR = 1.48; $P = 0.05$) and progression-free survival (PFS) (HR = 0.79; $P = 0.007$). Furthermore, in primary breast cancer patients treated with adjuvant tamoxifen, SIAH2 predicted metastasis-free survival (MFS) (HR = 0.73; $P = 0.005$). In vitro experiments showed that SIAH2 silencing in MCF7 cells resulted in resistance to ICI164.384-treatment when compared with mock silenced cells ($P = 0.008$). Interestingly, in ZR cells transfected with EGFR (ZR/HERc), SIAH2 expression was induced by E2 but downregulated by EGF. *Conclusion* In primary breast tumor specimens as well as in vitro low SIAH2 levels associated with resistance to endocrine therapy. Moreover, SIAH2 expression showed an opposite regulation by E2 and EGF.

Keywords SIAH2 · Ubiquitin E3 ligase · Endocrine therapy resistance · siRNA · Breast tumors

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Introduction

The selective estrogen receptor modulator (SERM) tamoxifen is one of the most important drugs for patients with breast cancer. However, in the advanced disease setting only half of breast cancer patients with ER expression respond to tamoxifen, underlining the need to understand mechanisms conferring its resistance against tamoxifen. One of the mechanisms is the activation of EGF Receptor (EGFR) and/or HER2 signaling pathway, as only 17% of these patients respond to tamoxifen [1]. Nevertheless, other resistance mechanisms remain to be identified.

Our profiling study of tamoxifen resistance revealed an 81-gene signature [2]. Of these SIAH2 was highly significant ($P = 0.001$) associated with response and progression-free

survival. SIAH2 belongs to the genes of the ER-positive epithelial breast cancer subtype and strongly associated with ER levels [3, 4], and it was overexpressed in malignant compared with normal breast tissue [5]. Moreover, SIAH2 (AC011317; 147 kb) sequence analysis revealed an estrogen response element (CAGGTCA NNNTGACCTG) [6] in the intron between exon 1 and 2.

SIAH2 and SIAH1 (OMIM 602213 and 602212) are human homologs of the Drosophila SINA proteins and belong to evolutionarily highly conserved ubiquitin E3 ligases [7, 8]. SIAH proteins interact and participate in ubiquitination and proteasome-dependent degradation of a subset of proteins. With respect to breast cancer, SIAH2 degrades proteins including HDAC3 and NCoR [9, 10], both participating in ER transcriptional co-repressor complexes implicated in tamoxifen repressive activity [11–13].

As yet, it is unclear whether SIAH2 has prognostic and/or predictive value in breast cancer or whether SIAH2 is functionally involved in therapy response. These questions are addressed in the current paper: response prediction was tested in 1205 breast cancer specimens while functional analysis of SIAH2 in therapy resistance was performed in vitro.

Patients and methods

Frozen breast tumor tissue specimens of female patients with primary operable breast cancer were analysed in this retrospective study (approved by the medical ethics committee of the Erasmus MC (MEC 02.953)). The present study, in which coded tumor tissues were used, was performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (<http://www.fmwv.nl>).

Based on previously described criteria and in accordance to the REMARK recommendations, tumor specimens and clinical data of 1205 patients were analysed [14–16]. About 511 patients (42%) underwent breast conserving lumpectomy and 694 patients (58%) modified mastectomy. Median follow-up time of patients alive was 91, range 3–248 months. Of the 720 patients with relapse (60%), 635 patients had distant disease recurrence and 522 patients were treated with systemic therapy for advanced disease. About 168 of these patients (32%) received chemotherapy and 354 patients (68%) were treated with hormonal therapy, of which 235 hormone naïve patients with ER + tumors (66%) received first-line tamoxifen monotherapy. Of these, 148 patients (64%) had clinical benefit from first-line tamoxifen monotherapy; 9 patients with complete and 35 partial response, and 104 patients showed stable disease longer than 6 months. Therapy failure occurred in 87 patients (36%);

72 had progressive disease and 15 had stable disease less or equal than 6 months. Other patient and tumor characteristics are listed in Table 1.

RNA isolation and quantitative real-time PCR

Tissue processing, RNA isolation, cDNA synthesis, quantitative real-time polymerase chain reaction and expression data generation were performed as described [14, 16].

Table 1 Associations of SIAH2 mRNA levels with clinicopathological factors

	No. of patients	%	Median	Δ^a	P^*
Total	1205	100	0.26	0.36	
Age in categories (y)					<0.001 ^e
≤40	150	12	0.19	0.26	
41–55	443	37	0.25	0.35	
56–70	405	34	0.27	0.38	
>70	207	17	0.32	0.51	
Menopausal status					0.17 ^f
Premenopausal	491	41	0.24	0.31	
Postmenopausal	714	59	0.27	0.42	
Tumor size					0.11 ^g
pT1, ≤2 cm	359	30	0.29	0.42	
pT2, >2–5 cm	702	58	0.24	0.35	
pT3, >5 cm + pT4	144	12	0.24	0.33	
Lymph nodes involved					0.12 ^g
0	603	50	0.23	0.36	
1–3	265	22	0.30	0.37	
>3	337	28	0.27	0.34	
Grade ^b					<0.001 ^h
Poor	675	56	0.22	0.32	
Good/moderate	160	13	0.33	0.47	
ER protein status ^c					<0.001 ^e
Low	332	28	0.12	0.10	
High	873	72	0.35	0.45	
PgR protein status ^{c, d}					<0.001 ^e
Low	419	35	0.15	0.16	
High	729	61	0.35	0.45	

^a Interquartile range (q75–q25); * Two-sided P -value

^b In 370 samples (31%) grade was unknown. The median SIAH2 expression levels in this subset was 0.31 ($\Delta = 0.45$)

^c Low and high steroid hormone receptor protein status as defined in the “Methods” section

^d In 57 samples protein levels of PgR were not determined

^e Spearman rank correlation

^f Mann–Whitney U test

^g Kruskal–Wallis test, a nonparametric test for trend was not applicable

^h Kruskal–Wallis test, followed by a nonparametric test for trend

qRT-PCR reactions were performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Nieuwekerk a/d IJssel, Netherlands) and a MX3000P Real-time PCR system (Stratagene, Amsterdam, Netherlands) according recommended protocols. An Assay-on-Demand kit (Hs00192581_m1; Applied Biosystems) was used to measure SIAH2 mRNA levels. Primer sequences for ER, PgR, EGFR, HER2, and reference genes PBGD, HPRT and B2 M have been described [14, 16, 17]. About 40 rounds of amplification were performed to generate Cycle threshold (Ct) values and to calculate mRNA expression levels. Gene expression levels were normalized against average expression levels of all reference genes in tumor samples, or HPRT levels in breast cancer cell lines.

Breast cancer cell lines cultures

Three human breast cancer cell lines MCF7, ZR-75-1, and ZR/HERc were studied. MCF7, estrogen sensitive, was cultured in RPMI 1640 containing 10% foetal calf serum. The estrogen-dependent ZR-75-1 and its EGFR-transfectant, ZR/HERc, were maintained in RPMI 1640, 10% bovine calf serum and 1 nM 17 β -estradiol as described previously [17]. Cell lines were grown in different culture conditions in short- (2, 4 and 6 h) and long-term (96, 168 and 192 h) experiments. Charcoal treated serum and phenol red-free medium were used to obtain E2-depleted culture conditions. Medium was supplemented with previously described compounds [17]: E2 (10 nM), ICI164,384 (100 nM), EGF (10 ng/ml) or a combination, or with ethanol vehicle alone as indicated in the legends. In vitro, to evaluate only pure anti-E2 effects, the selective estrogen receptor degrader (SERD) ICI164,384 was used instead of tamoxifen. Throughout an experiment, culture medium was renewed every 3 days and at the end, cells were lysed, and RNA and protein isolated using the MirVana Paris kit according the manufacturer's protocol (Ambion, Foster City, CA).

Western blotting

Protein samples were separated on 4–12% SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto PVDF membranes. Diluted antibodies were used against SIAH2 (polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA (1:100); and monoclonal Sigma, St. Louis, MO (1:625)), and GAPDH (monoclonal, (1:500)). Antibodies were detected with HRP- or HRP-polymer (DAKO Real Envision, DAKO, Diagnostica GmbH, Hamburg, Germany) labeled secondary antibodies and chemiluminescent reagents (ECL-kit, Pierce, Rockford, IL). The Scanalytics One-D program (Alpha Innotech Ltd., Cannock, UK) was used for quantification of protein bands.

RNA interference

Small interfering RNAs (siRNA) targeting SIAH2 and ER mRNA were obtained from Qiagen, Inc. (Venlo, Netherlands) and used according recommended instructions. Briefly, 5 nM of two SIAH2-specific siRNAs with target sequences ACCCGGAGTGCTTATCTTAAA and TTGG GTGGATTGCCTAGGAAA were pooled and introduced via inverse transfection into MCF7 using HiPerfect transfection reagent (Qiagen). A validated ER-specific siRNA (SI02781401) was used to investigate the ER signaling pathway. As control, MCF7 cells were transfected with non-specific (NS) control pool siRNA (Qiagen).

Experiments were performed in triplicate at different time-points in 96 wells plates. Per condition 8 wells were used and pooled for mRNA and protein analysis. In each well, 5000 cells were transiently transfected, cultured for 4 days, harvested, again transfected with siRNAs, and subsequently grown for 4 days in standard culture medium supplemented with 100 nM ICI164,384 or ethanol vehicle as control. Cell numbers were counted with a coulter counter to assess effects of SIAH2 silencing on cell proliferation at day 4 and 8 and at day 8 for response to 4 days of ICI164,384-treatment.

Data analysis and statistics

Relationships of SIAH2 with patient and tumor characteristics were investigated using nonparametric methods, i.e., Spearman rank correlations for continuous variables and Wilcoxon or Kruskal–Wallis test for ordered variables. Cox uni- and multi-variate regression analysis was applied to compute the hazard ratio (HR) using the occurrence of a distant metastasis for metastasis-free survival (MFS), survival for overall (OS), and disease progression for progression-free survival (PFS) as endpoints, as described previously [14, 18]. For those subsets that were analyzed in multivariate analysis, the proportional hazard assumption for SIAH2 was not violated in univariate analysis. Logistic regression analysis was performed to calculate the odds ratio (OR) that defines the relation between expression levels and type of response. Both HR and OR were calculated on transformed variables to reduce skewness and were represented with their 95% confidence interval (95% CI). For SIAH2 a box–cox transformation was applied. Survival curves were generated using the method of Kaplan and Meier and a log rank test was used to test for differences.

A median cutoff point as well as an optimized cutoff point for SIAH2 was considered justified when the test for trend as continuous variable was statistically significant. To define an optimized cutoff point, isotonic regression analysis was used to discover points where monotonic relationships between measured level and hazard ratio showed distinct changes [19]. The cutoff point with the

largest statistically significant change in hazard ratio corrected for multiple testing was used.

Computations were performed with the STATA statistical package, release 9.2 (STATA Corp., College Station, TX). All *P*-values were two-sided and *P* < 0.05 was considered statistically significant.

Results

Associations of SIAH2 mRNA expression levels with clinicopathological factors

Associations of SIAH2 levels with clinicopathological factors in all patients (*n* = 1205) are summarized in Table 1. SIAH2 levels were significantly associated with age, grade, ER and PgR protein. At mRNA level, a significant (*P* < 0.001) positive correlation between SIAH2 and ER ($r_s = 0.63$), PgR ($r_s = 0.56$) and HER2 ($r_s = 0.10$) was detected whereas a significant inverse correlation (*P* < 0.001) between SIAH2 and EGFR mRNA levels ($r_s = -0.25$) was observed.

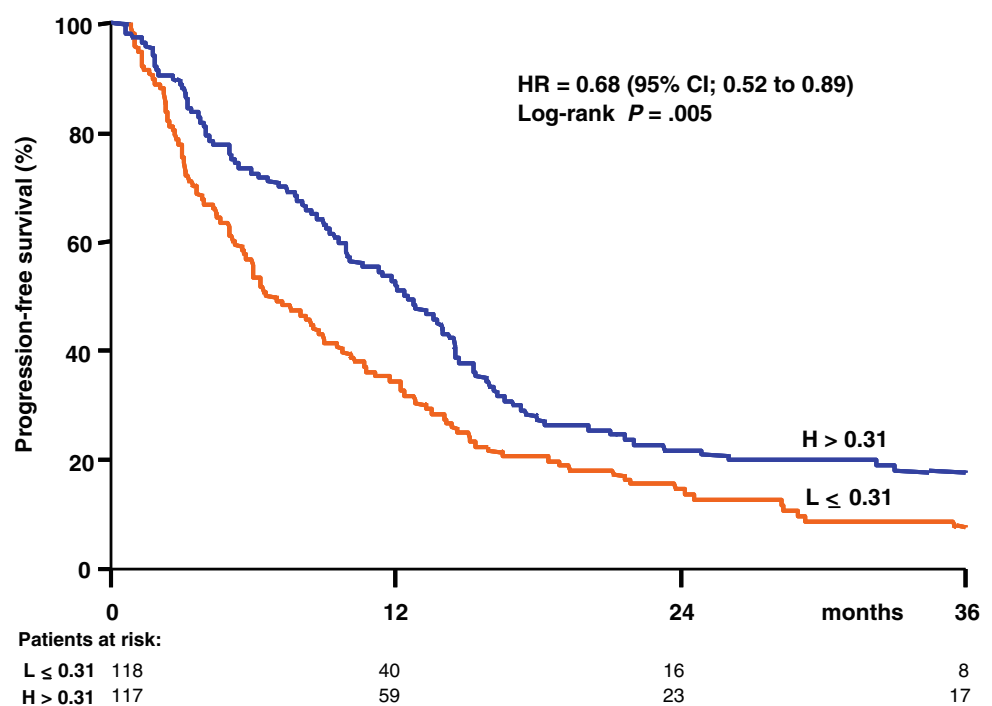
SIAH2 levels and clinical outcome in different patient subsets

In all 1205 tumors, high SIAH2 levels were significantly associated with a favorable MFS (HR = 0.85 [95% CI: 0.80–0.92]; *P* < 0.001) and OS (HR = 0.82 [95% CI: 0.76–0.88]; *P* < 0.001). In 603 lymph node-negative (LNN) patients who did not receive (neo)-adjuvant systemic

therapy, SIAH2 expression did not associate with tumor aggressiveness, since it did not significantly predict MFS (HR = 0.98 [95% CI: 0.88–1.09]; *P* = 0.72) nor OS (data not shown). In 602 lymph node-positive (LNP) patients, higher SIAH2 levels were significantly associated with a favorable MFS (HR = 0.70 [95% CI: 0.63–0.77]; *P* < 0.001) and OS (HR = 0.66 [95% CI: 0.60–0.73]; *P* < 0.001). In ER-positive tumors of LNP patients treated with adjuvant tamoxifen monotherapy (*n* = 139), higher SIAH2 levels in continuous analysis were significantly related with a longer MFS (HR = 0.69 [95% CI: 0.57–0.84]; *P* < 0.001) and OS (HR = 0.73 [95% CI: 0.60–0.88]; *P* = 0.001).

For a direct evaluation of therapy effects, i.e. the size of metastasis and/or the occurrence of new lesions, SIAH2 expression was related with type of response and PFS in patients with advanced disease who were treated with first-line systemic monotherapy. In ER-positive tumors of patients who received first-line tamoxifen monotherapy (*n* = 235), SIAH2 expression as continuous variable was significantly associated with clinical benefit (OR = 1.71 [95% CI: 1.25–2.34]; *P* < 0.001) and PFS (HR = 0.74 [95% CI: 0.63–0.86]; *P* < 0.001). When dichotomized at the median expression level (0.31), high SIAH2 levels were significantly associated with clinical benefit (OR = 2.32 [95% CI: 1.35–4.00]; *P* = 0.002) and long PFS (HR = 0.68 [95% CI: 0.52–0.89]; *P* = 0.005). Moreover, Kaplan–Meier survival analysis for PFS showed a median difference of 6 months between patients with high against low SIAH2 levels (Fig. 1). In addition, an optimized cutoff point (expression level: 0.11)

Fig. 1 Relationship of dichotomized SIAH2 levels with progression-free survival (PFS) in 235 ER-positive tumors of patients with advanced disease treated with first-line tamoxifen therapy. Median cutoff point (0.31) was used to dichotomize SIAH2 expression levels



determined with isotonic regression analysis, resulted in a much stronger association between high SIAH2 levels (>0.11 , ($n = 209$)) and clinical benefit (OR = 3.48 [95% CI: 1.47–8.27]; $P = 0.005$) and long PFS (HR = 0.39 [95% CI: 0.26–0.60]; $P < 0.001$).

SIAH2 and tamoxifen monotherapy: multivariate analysis

SIAH2 showed a significant association with benefit of tamoxifen monotherapy in the adjuvant as well as in the advanced disease setting. Since the proportional hazard assumption was not violated, SIAH2 as continuous variable was compared in multivariate analysis with established prognostic and predictive factors, respectively. In the 139 ER-positive tumors from LNP patients treated with tamoxifen in the adjuvant disease setting, SIAH2 was independent from the prognostic factors and associated significantly with MFS (HR = 0.73 [95% CI: 0.58–0.91]; $P = 0.005$). Moreover, in the 235 ER-positive tumors of patients with advanced disease, high SIAH2 levels were independently associated with a favorable disease outcome (Table 2). Interestingly in this subset of patients, SIAH2 had an even stronger association than ER for PFS and than PgR for disease outcome.

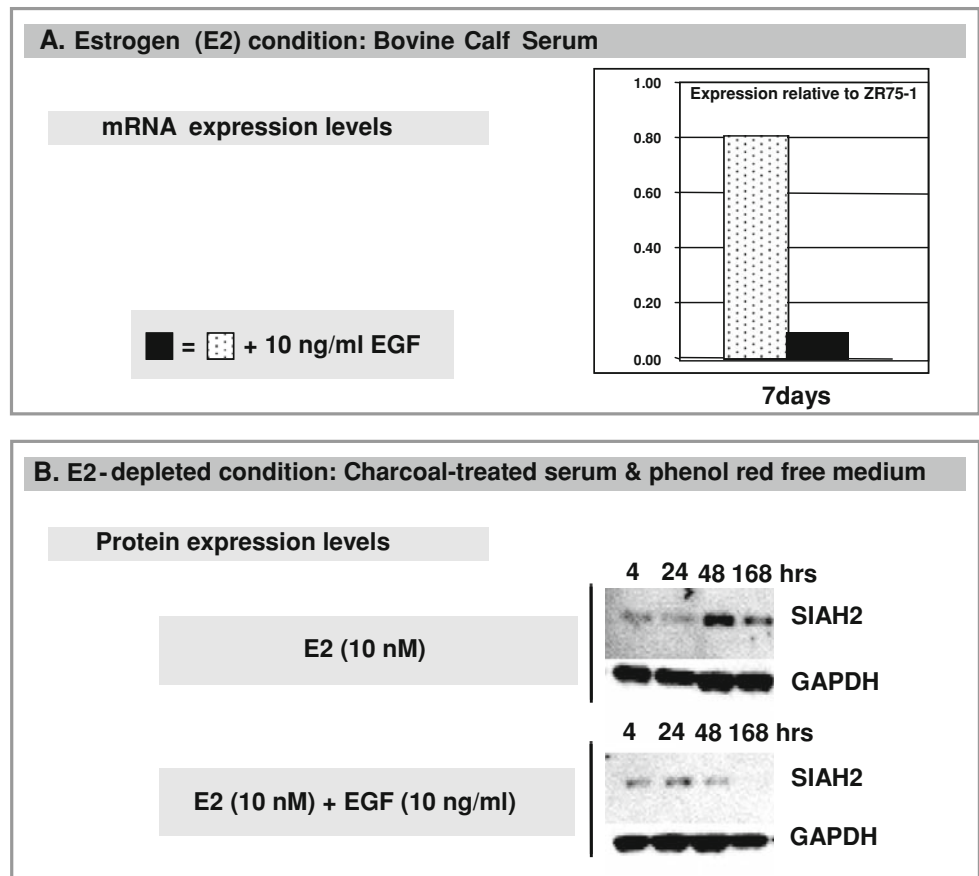
Regulation of SIAH2 expression by ER and EGFR

Our in vitro studies in ER-positive human breast cancer cell lines MCF7, ZR-75-1 and ZR/HERc confirmed that SIAH2 expression was induced by E2 (1 and 10 nM) while repressed by the SERD ICI164,384 (100 nM and 1 μ M) (data not shown). Addition of 10 nM E2 to ZR-75-1 and ZR/HERc cells grown in E2-depleted culture conditions resulted within 2 h in a 5-fold increase in SIAH2 mRNA levels, which was reduced to almost basic SIAH2 levels by 1 μ M ICI164,384-treatment. Silencing of ER in MCF7 resulted also in decreased SIAH2 protein levels (data not shown). Because of the observed inverse correlation between SIAH2 and EGFR mRNA expression in breast tumor specimens, we investigated the regulation of SIAH2 expression by EGFR. Interestingly, addition of 10 ng/ml EGF to ZR/HERc cells (ZR75 transfected with EGFR) in long-term culture conditions resulted in decreased SIAH2 expression at mRNA (Fig. 2a) and protein level. Moreover, in the E2-depleted culture experiment, SIAH2 expression was induced by 10 nM E2 but this E2-action was opposed by addition of 10 ng/ml EGF resulting in a 2-fold reduction of SIAH2 protein levels after 7 days culturing (Fig. 2b). Additionally, in silico analysis of public datasets was performed with OncoPrint (www.oncoPrint.org). This analysis showed a significant

Table 2 Multivariate analysis for type of response and progression-free survival (PFS) of SIAH2 as continuous variable in estrogen receptor-positive tumors from 235 patients whose recurrence was treated with first-line tamoxifen monotherapy

Factor of base model	No. of patients	%	Clinical benefit			PFS		
			OR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>
Age (years)								
≤56	91	39	1.00			1.00		
56–70	84	36	1.13	0.41–3.13	0.81	0.70	0.44–1.12	0.14
>70	60	25	1.25	0.43–3.68	0.68	0.60	0.36–0.99	0.042
Menopausal status								
Premenopausal	61	26	1.00			1.00		
Postmenopausal	174	74	0.91	0.33–2.47	0.85	1.78	1.11–2.84	0.016
Disease-free-survival								
≤1 year	38	16	1.00			1.00		
1–3 years	118	50	6.83	2.84–16.40	<0.001	0.41	0.28–0.62	<0.001
>3 years	79	34	9.40	3.61–24.45	<0.001	0.30	0.19–0.46	<0.001
Dominant site of relapse								
Soft tissue	23	10	1.00			1.00		
Bone	115	49	0.39	0.13–1.19	0.10	1.47	0.88–2.46	0.14
Viscera	97	41	0.51	0.16–1.59	0.24	1.50	0.89–2.52	0.13
Adjuvant chemotherapy								
No	190	81	1.00			1.00		
Yes	45	19	1.73	0.72–4.14	0.22	1.03	0.70–1.54	0.87
ER mRNA	235	100	1.24	1.07–1.44	0.005	0.91	0.85–0.97	0.008
PgR mRNA	235	100	1.05	0.88–1.26	0.56	0.92	0.85–1.00	0.051
SIAH2 mRNA	235	100	1.48	0.99–2.21	0.053	0.79	0.67–0.94	0.007

Fig. 2 Expression of SIAH2 in ZR/HERc cells: Regulation by estrogen (E2) and epidermal growth factor (EGF), in E2 containing (a) or E2-depleted culture conditions (b). Data shown are of two independent cell culture experiments



downregulation of SIAH2 mRNA levels in the dataset of Creighton et al. [20]. In the latter study the epidermal growth factor signaling pathway was activated in MCF7 cells via transfection of EGFR, MEK, or HER2 (Fig. 3). Thus, ER and EGFR have opposing effects on SIAH2 levels, i.e., E2 induces whereas EGF reduces SIAH2 expression.

SIAH2 and endocrine therapy resistance

To investigate whether SIAH2 is directly involved in endocrine therapy resistance, SIAH2 expression in MCF7 was downregulated with siRNAs and/or 100 nM ICI164,384. SIAH2 silencing resulted in approximately 85% decrease in mRNA expression levels in untreated and ICI-treated cells (Fig. 4a). As expected SIAH2 mRNA levels were downregulated (40%) by ICI164,384.

For each condition cell numbers were counted. SIAH2 silencing by itself did not affect proliferation of MCF7 cells (Fig. 4b), however, when treated with ICI164,384 for 4 days, a significant decrease (25%; $P = 0.001$) of mock transfected cell numbers was observed compared with the untreated condition. Interestingly, we observed only a 5% difference in cell number between the ICI-treated versus untreated SIAH2 transfected cells (Fig. 4b). Thus downregulation of SIAH2

expression in MCF7 resulted in resistance to ICI164,384 treatment.

Discussion

In the present study we have evaluated the prognostic and predictive value of SIAH2 in a large number of primary breast tumors and have assessed its role in endocrine therapy resistance in vitro. We show that SIAH2 is an independent predictor of disease outcome after tamoxifen therapy and our in vitro studies demonstrated that SIAH2 silencing resulted in resistance to anti-estrogens.

Our study shows that SIAH2 expression levels in LNN patients are not associated with prognosis, i.e., tumor aggressiveness in LNN patients. On the other hand, in LNP patients treated with adjuvant tamoxifen monotherapy an association of SIAH2 with MFS and OS was observed. This might be based on the tumor's response to endocrine therapy, its intrinsic aggressiveness, or both. However, in uni- and multi-variate analysis SIAH2 expression as continuous variable associated significantly with a favorable disease outcome in ER-positive tumors after first-line tamoxifen therapy.

Fig. 3 In silico analysis of public datasets available in ONCOMINE (www.oncofine.org) showed significant downregulation of SIAH2 mRNA levels in the dataset of Creighton et al. [20] after transfection of MCF7 cells with EGFR (a), MEK (b) or HER2 (c)

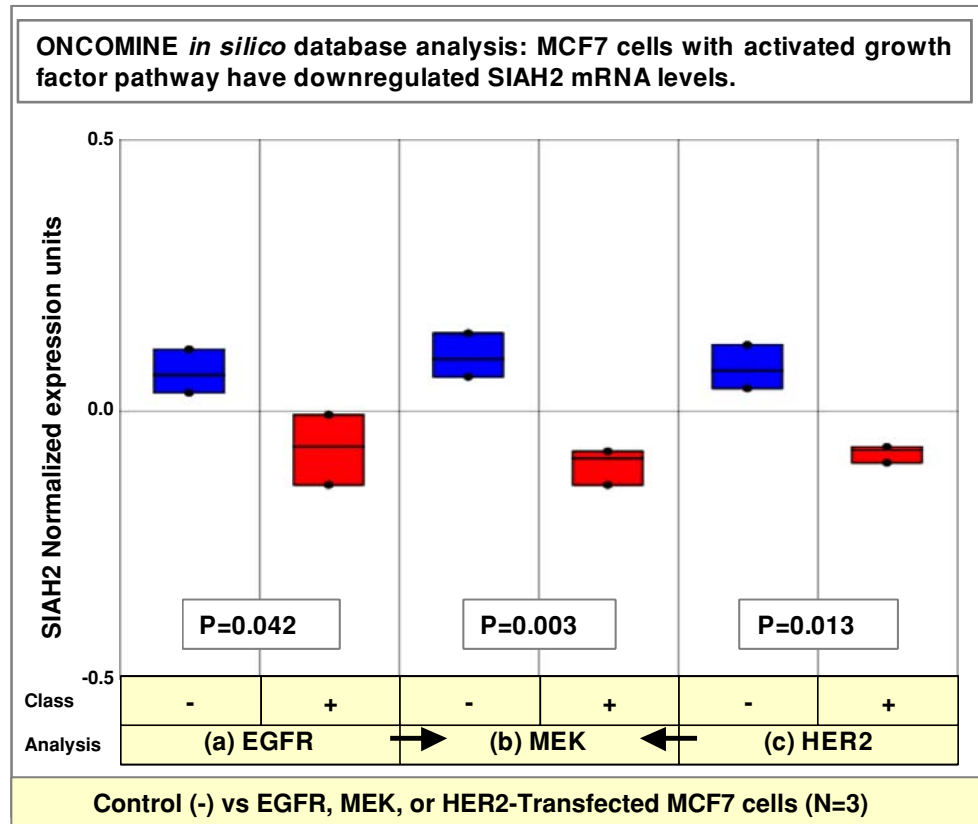
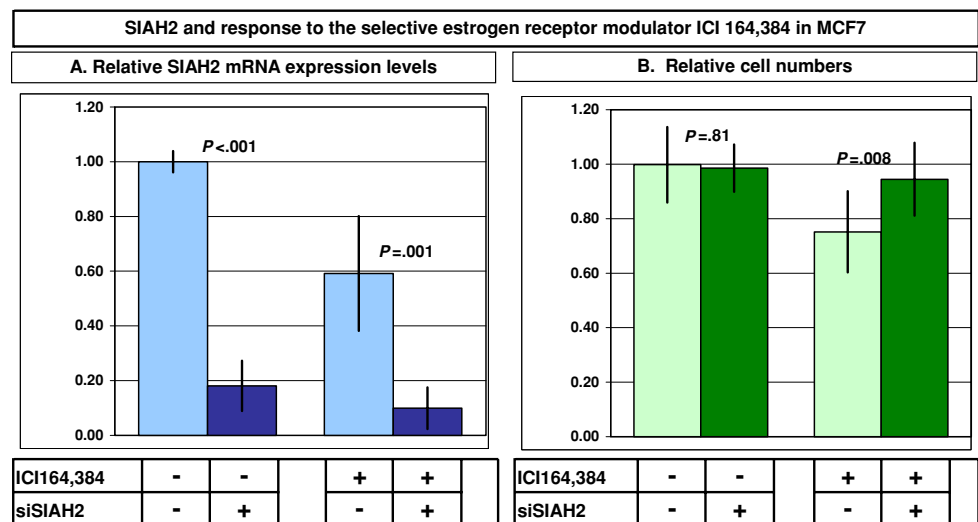


Fig. 4 SIAH2 silencing (siSIAH2) in MCF7 and response to ICI164,384 treatment. SIAH2 mRNA levels (a) and cell numbers (b) measured in siSIAH2 and/or ICI164,384 treated MCF7 cells. Bars represent mean ± sd of duplicate (a) and triplicate (b) measurements expressed as fold-difference compared with untreated MCF7 cells obtained from 3 time-point independent culture experiments



Our findings in primary breast tumors and cell lines indicate a relation between SIAH2 and ER and confirmed results of Frasar et al. [9, 21] that SIAH2 expression is upregulated by E2 and downregulated by SERMs and SERDs. Thus SIAH2 expression, like PgR, might be a marker of a functional (genomic) ER signalling pathway. Interestingly, in our primary breast tumors a significant inverse correlation between SIAH2 and EGFR mRNA expression was observed and, in vitro, we demonstrated

that addition of EGF to ZR/HERc cells resulted in a downregulation of SIAH2 levels. Results of the in silico data analysis [20] and supplemental data of Massarweh et al. [22] also showed that SIAH2 mRNA expression levels were downregulated in EGFR transfected MCF7 cells when treated with EGF. This indicates that loss of SIAH2 expression might be indicative of an active EGF/EGFR signaling pathway. Furthermore, high HER2 and EGFR expression levels have already been related with

resistance to tamoxifen therapy and a crosstalk between ER and the growth factor pathway have been suggested in endocrine-resistant breast cancer [12, 23, 24].

We hypothesize that SIAH2 expression levels may guide us as a biomarker for response to endocrine therapy and that its levels are upregulated by ER but downregulated by EGFR. Depending on the SIAH2 levels in untreated primary tumors due to this ER/EGFR regulation, a metastasis is proposed to be sensitive (high SIAH2 levels, due to ER) or resistant (low SIAH2 levels, due to EGFR) to first-line tamoxifen therapy.

To address whether SIAH2 expression deficiency in (untreated) primary tumor cells results in endocrine therapy resistance, we have downregulated SIAH2 expression with targeted siRNAs in the (anti-)estrogen sensitive breast cancer cell line MCF7 and subsequently treated these cells with ICI164,384. Indeed, our results indicate that an initial loss of SIAH2 is associated with a significant decreased sensitivity to the pure anti-estrogen ICI, already after 4 days.

Since SIAH2 is involved in the proteasomal degradation of proteins, its silencing evokes protein accumulation. Frasar et al. [9] and our pilot studies (data not shown) showed that SIAH2 silencing caused NCoR and HDAC3 accumulation, respectively, both proteins being part of an ER-associated transcriptional corepressor complex which are necessary for the inhibitory effect of tamoxifen [11, 13]. Therefore, it was anticipated that NCoR and HDAC3 accumulation, as a result of SIAH2 silencing, would give an increased sensitivity to ICI164,384. Since we observed low SIAH2 levels in tamoxifen resistant tumors and a decreased sensitivity to ICI164,384 after SIAH2 silencing in MCF7, the outcome of our study is in contrast to this hypothesis and might even be counterintuitive. We propose that due to initial low SIAH2 levels no noteworthy downregulation of SIAH2 and subsequent additional accumulation of NCoR and HDAC3 proteins by anti-estrogens might occur. As a result, a shift to corepressor instead of coactivator protein complex binding to ER will not take place and tumor cells will continue to proliferate. Because our study showed that SIAH2 expression levels are downregulated by EGF, it may be worthwhile to explore whether or not the outcomes of endocrine treatment are improved by the addition of EGFR inhibitors such as lapatinib, erlotinib, gefitinib, and cetuximab in this particular population of patients with ER-positive tumors and low SIAH2 levels. A recent presurgical study reported by Guix et al. [25] supports above assumption. The authors showed that erlotinib treatment inhibited proliferation and postreceptor signalling pathways in ER-positive tumors.

In conclusion, we showed that SIAH2 is up- and downregulated by ER and EGFR, respectively. Furthermore, in tamoxifen treated breast cancer patients low SIAH2 levels

were associated with short MFS in the adjuvant setting and with resistance to tamoxifen monotherapy in the advanced setting. In line with this, our in vitro studies demonstrated a decreased sensitivity for ICI164,384 after silencing of SIAH2 expression.

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