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The expression of the ubiquitin ligase SIAH (seven in absentia homolog) 2 is mediated through gene copy number in breast cancer and is associated with a basal-like phenotype and p53 expression

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Abstract

Introduction: *SIAH2* plays a significant role in the hypoxic response by regulating the abundance of HIF-1alpha, however, its role in breast carcinoma is unclear. We investigated the frequency and expression pattern of *SIAH2* in two independent cohorts of sporadic breast cancers.

Methods: Immunohistochemical evaluation of *SIAH2* protein expression was conducted in normal breast tissues and in tissue microarrays comprising ductal carcinoma *in situ* and a cohort of invasive breast carcinomas. Correlation analysis was performed between *SIAH2* and clinicopathological variables and intrinsic breast cancer subgroups and validated on a cohort of 293 invasive ductal carcinomas. Promoter methylation, gene copy number and mRNA expression of *SIAH2* was determined in a panel of basal-like tumors and cell lines.

Results: There was a significant increase in nuclear *SIAH2* expression from normal breast tissues through to DCIS and progression to invasive cancers. A significant inverse correlation was apparent between *SIAH2* and ER and PR and a positive association with tumor grade, HER2, p53 and intrinsic basal-like subtype. A logistic regression confirmed the significant positive association between *SIAH2* expression and basal-like phenotype. No *SIAH2* promoter methylation was identified, yet there was a significant correlation between *SIAH2* mRNA and gene copy number. *SIAH2* positive tumors were associated with a shorter relapse-free survival in a univariate but not multivariate analysis.

Conclusions: *SIAH2* expression is upregulated in basal-like breast cancers via copy number changes and/or transcriptional activation by p53 and is likely to be partly responsible for the enhanced hypoxic drive through abrogation of the prolyl hydroxylases.

Introduction

Hypoxia in breast cancer has profound effects on tumor biology that is reflected in a poor prognosis and resistance to both chemotherapy and radiotherapy in patients [1]. Hypoxia-inducible factor-1 is critical to the hypoxic response, being a transcription factor that through binding to hypoxia response elements in the promoters of genes results in expression of proteins involved in angiogenesis (vascular endothelial growth factor, VEGF), glucose metabolism (GLUT1), metastasis (CXCR4, SDF1), cell survival and proliferation.

HIF-1 is a dimer consisting of a constitutively expressed aryl nuclear translocator (ARNT) or HIF-1β and a hypoxia-inducible HIF-1α. The levels of HIF-1α are tightly regulated by three prolyl hydroxylases. In the presence of molecular oxygen these enzymes hydroxylate the prolyl residues 402 and 564 in the oxygen dependent domain of HIF-1α resulting in conformational change and recognition by the VHL protein that leads to its ubiquitination and degradation via the proteasome. In contrast, under hypoxia, the prolyl hydroxylases have limited molecular oxygen and are therefore less effective, which enables HIF-1α stabilization, translocation to the nucleus and initiation of gene transcription that benefits the tumor.

Siah (seven in absentia homolog)2 is one of a family of RING-domain proteins which act alone or as components of ubiquitin ligase complexes target proteins for proteasomal degradation [2]. Siah proteins can interact with many intracellular pathways including the scaffold proteins, transcriptional repressors and nuclear receptor co-repressors (NCoR) and β-catenin. Siah proteins are also involved in hypoxia signaling, via regulation of HIF-1α [3] through the targeted degradation of prolyl hydroxylases under hypoxic conditions. Indeed, *SIAH2* knockout mice have a delayed and abrogated response to hypoxic conditions that is mediated through reduced levels of HIF-1α [3] [4]. These data suggest that Siah proteins may significantly alter HIF signaling through modulation of the prolyl hydroxylases.

Although the role of HIF has been documented in breast cancer [5, 6] there is no data

on the expression of *SIAH2* in this disease. We have therefore investigated *SIAH2* expression in breast cancer in two independent cohorts. Our aims were to (1) document the pattern and level of *SIAH2* expression in breast cancer, (2) correlate expression with conventional clinicopathological factors, (3) investigate associations of *SIAH2* expression with intrinsic subtypes of breast cancer and (4) determine the effect of *SIAH2* expression on relapse-free survival (RFS).

Materials and methods

Patients

The flow of patients through the study according to the REMARK criteria is listed in Supplementary Table 1 in Additional File 1(24). The first cohort was derived from the Department of Pathology, Peter MacCallum Cancer Centre, and comprised 120 cases with full clinicopathological characteristics but without survival data. The second cohort was from the Garvan Institute, Sydney, and comprised 293 cases with full clinicopathological characteristics including survival data [7]. In total 439 invasive cancers with clinicopathological data and follow up were available for study. Of these 439 cases, 61 cases were excluded due to inadequate tumor tissue on the array. The final cohort of invasive cancers was composed of 378 cases (246 cases with survival data). 80 cases of pure DCIS were obtained from the John Radcliffe Hospital, Oxford, UK, of which 54 had DCIS on the TMAs for staining and clinical data available. Ten cases of normal post menopausal breast tissues from mammoplasties were also collected. This study has Ethics Committee approvals (numbers 00/81, 03/90, 09/36, JRC02.216, HREC SVH H94/080 and H00/36). All patients had operable breast carcinomas and were not diagnosed with distant metastatic disease at the time of presentation. Information regarding patient characteristics, including age, tumor size, grade, histology and nodal status were collected from the clinical and pathological records. The median age of patients included in this study was 54 years (range 24 to 87 years). Ninety

three percent of tumors were invasive ductal of no otherwise specified (NOS) type, 3% were invasive lobular carcinoma and 4% were of other histological types (data was unavailable for 2 cases). Median tumor size was 20 mm and the median tumor grade was 2. Forty one percent of patients had nodal disease. Sixty-nine percent of tumors were ER positive and 14% were HER2 positive. Patients less than 50 years of age with node positive, ER negative tumors or tumors larger than 3cm received adjuvant chemotherapy (cyclophosphamide, methotrexate and 5-fluorouracil (CMF) or adriamycin and cyclophosphamide (AC). Patients with hormone responsive tumors who were more than 50 years of age received 5 years of endocrine therapy. Patients were followed up for a median period of 58.1 months. During this time, in the 100 patients from the invasive cohort 2 developed recurrence and 86 deaths were considered breast-cancer related.

Immunohistochemistry

TMAs were constructed from 1 mm diameter (invasive cancers) or 2 mm cores (DCIS). Sections of 4 μm thickness were used for immunostaining. TMA sections were dewaxed and antigen retrieval performed in 10mM sodium citrate pH 6 in a pressure cooker for 3 minutes. Sections were then treated with 3% H_2O_2 for 5 min to remove endogenous peroxides, washed and incubated with a SIAH2 antibody (Novus Biologicals®, NB110-88113) [8, 9] at 1:50 dilution for 90 minutes at room temperature. Application of the peroxidase-coupled Mouse ImmPRESS (Vector Laboratories) detection reagent was then used and staining was visualized with DAB+ (Dako Australia). Sections were counter-stained with hematoxylin to visualize nuclei. To analyze the expression of SIAH2 in breast cancer progression, we assessed expression using a combination of both intensity and proportion of cells expressing SIAH2 (26). Normal breast epithelium and tumors were scored for intensity (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining) and the percentage of cells (0 = no cells staining positive, 1 = < 10% cells staining positive, 2 = 10% to 50% positive cells, 3

= 51% to 80% positive cells, 4 = > 80% positive cells) as previously reported [10]. The scores for intensity and percentage of positive tumor cells were added together to give a maximum score of 7. A cut off of >2 (the median) was used to define two approximately equal size groups of patients for subsequent statistical analyses.

ER, HER2, EGFR and CK5/6 staining were used to classify tumors into 4 intrinsic subgroups: the basal group (ER negative, HER2 negative, CK5/6 and/or EGFR positive), luminal group (ER positive, HER2 negative), HER2 group (HER2 positive) and the negative (null) group (ER negative, HER2 negative, CK5/6 negative and EGFR negative) [11].

Analysis of SIAH2 Methylation

DNA from a separate series of 60 breast carcinomas (John Radcliffe Hospital, Oxford) and 5 normal breast tissues (Peter MacCallum Cancer Centre), comprising all breast phenotypes (50 ER positive, 10 ER negative)(also see Supplementary Table 2 in Additional File 2) and DNA was also obtained from the breast cancer cell lines MCF10A, MCF7, BT20, SkBr3, Hs578T, T47D, MDA-MB 157, MDA-MB 468, MDA-MB 453, MDA-MB 231, MDA-MB 361, BT483 and ZR75. Bisulfite modified (EpiTect® Bisulfite kit, Qiagen, Hilden, Germany) DNAs were assessed for *SIAH2* methylation using methylation-sensitive high resolution melting (MS-HRM)[12]. The MS-HRM primers for *SIAH2*: 5'-TAGAAGCGGGTGGGTTAGGGTTT-3' (forward) and 5'-CTAATACACTCCGCAACCCCC-3' (reverse) amplified a region corresponding to GenBank access number AC011317.23 nucleotides 105279-105409 which contains 17 CpGs. PCR was performed in a final volume of 20 μl. The PCR reaction mixture consisted of 1x PCR buffer (Qiagen), 2.5 mM of MgCl2 , 200 μM of each deoxyribonucleotide triphosphate, 200 nM of the forward primer, 200 nM of the reverse primer, 5 μM of SYTO9 intercalating dye (Invitrogen), 0.5 U of HotStarTaq DNA polymerase (Qiagen), and 1 μl (theoretical amount 10 ng) of bisulphite modified DNA. The PCR amplification was performed with an

activation step of 15 min at 95°C, followed by 50 cycles of 10 s at 95°C, 10 s at an annealing temperature of 66°C, 20 s at 72°C for extension; and one denaturation step of 1 min at 97°C. High resolution melting was directly performed after PCR amplification: PCR products were denatured at 97°C for 1 min then cooled to 75°C with temperature rising by 0.2°C per second to 97°C and holding for 1 sec after each stepwise increment. Methylated sequences could be identified by their increased melting temperature [12]. In each assay, fully methylated, peripheral blood DNA (unmethylated), different methylation percentage dilution standards and non-template controls were included as controls and standards. All assays were performed in duplicate.

Copy number analysis of *SIAH2*

A previous study analyzed both gene expression and copy number variation using the Illumina human-6 beadarrays and the CNV370 SNP arrays respectively in a cohort of familial tumors [13]. These familial tumors were known to be *BRCA1*, *BRCA2* or non-*BRCA1/2* tumors and in this previous study the familial tumors were classified into one of the breast tumor subtypes: basal-like, luminal A, luminal B, HER2+ and normal-like. These data were used to determine the expression of *SIAH2* and its copy number status in 15 basal-like tumors. The copy number of *SIAH2* in each tumor was inferred from the average logR value of 8 SNPs, which were within the *SIAH2* open reading frame (n=3) or in the sequence flanking the gene (n=5).

Results

SIAH2 expression in normal breast, *in situ* and invasive breast carcinomas.

SIAH2 expression was identified in the nuclei of occasional cells within luminal layer of ducts and acini in normal breast tissues in 3/10 (30%). This was usually mild to moderate intensity and when stratified using the cut-off used for the tumors all were considered

negative for SIAH2 (Figure 1A). Expression of SIAH2 was observed in the nuclei of 7/54 (13%) DCIS cases. The staining was generally of a moderate to strong intensity with a homogenous distribution (Figure 1B&C). There was a non-significant increase in SIAH2 from the transition of normal to *in situ* disease (p=0.13) and a significant increase in SIAH2 from *in situ* to invasive breast carcinoma (p=0.0006)(Table 1)(Figure 1D and 2).

Association between SIAH2 protein expression and clinicopathological characteristics in DCIS

There was no significant correlation between SIAH2 expression with nuclear grade, presence of necrosis, age, ER, PR, EGFR or HER2 (all p>0.05) or intrinsic phenotypes in DCIS (p = 0.471) (Supplementary Table 3 in Additional File 3).

Correlation between SIAH2 **protein expression with clinicopathological characteristics and intrinsic subtypes in invasive cancer**

In the primary cohort, there was a significant inverse correlation between SIAH2 protein expression and ER (p<0.0001), PR (p=0.011) and a positive association with tumor grade (p<0.0001) and intrinsic subtype (p=0.028) but there was no association with patient age, tumor size, lymph node status or HER2 (all p>0.05)(Supplementary Table 4 in Additional File 4). In the validation cohort, there was a significant inverse correlation between SIAH2, ER (p<0.0001) and PR (p<0.0001) and a significant positive association with tumor grade (p<0.0001), patient age (p=0.009), HER2 (p=0.007) and intrinsic subtype (p<0.0001), but not with tumor size or lymph node status (p>0.05)(Supplementary Table 5 in Additional File 5). In the combined cohort there was a significant inverse correlation between SIAH2 and ER (p<0.0001), PR (p<0.0001) and a positive correlation with tumor grade (p<0.0001), HER2 (p=0.007), p53 (p<0.001) and intrinsic subtype (p<0.0001) but not with patient age, tumor size or lymph node status (p>0.05)(Table 2). The significant associations between SIAH2

expression grade and intrinsic subgroups were confirmed in a multivariate analysis of tumor phenotype, age, grade and lymph node status with the basal-like phenotype being over 5 times more likely to express SIAH2 than luminal tumors (Table 3)(p=0.015), which was also observed in the combined cohort (p=0.042).

Promoter methylation of *SIAH2* in cell lines and tumors

To assess whether *SIAH2* expression in tumors is modulated by promoter methylation, a CpG islands were identified in the promoter region of *SIAH2* and MS-HRM primers were designed to cover the CpG rich area of the promoter region of *SIAH2*. Five normal breast tissues, 60 breast carcinomas and 13 breast cancer cell lines were screened for methylation of *SIAH2* but no promoter methylation was detected in these cancer cell lines and samples as can be seen by the absence of altered methylation profiles (Supplementary Figure 1 in Additional File 6).

Correlation of gene expression and relative copy number of *SIAH2* in basal-like tumors

A cohort of familial tumors, which included 15 basal-like tumors was previously analyzed by gene expression and copy number analysis [13]. The 15 basal-like tumors showed a significant correlation between *SIAH2* expression and estimated copy number (r = 0.675, p=0.003)(Figure 3). Two of the 15 basal-like tumors showed a copy number gain (copy number of 3) and a further 3 of 15 basal-like tumors showed LOH at this region. In contrast 15 non basal-like tumors (10 luminal A, 4 luminal B, 1 normal-like tumor) did not show copy number change at this region.

Relationship between SIAH2 expression relapse free and overall survival

There was no correlation present between SIAH2 expression and overall relapse free survival in DCIS only patients (p=0.68). Although there was a significantly shorter relapse-free survival in all patients with invasive carcinomas stratified by SIAH2 (p=0.002)(Figure 4), no

significant association with relapse-free survival was observed in a univariate analysis in different breast cancer intrinsic groups stratified by SIAH2 (data not shown). There was also no significant association between SIAH2 in invasive carcinomas of all patients and relapse-free survival in a multivariate analysis (Table 4).

Discussion

Hypoxia is a pivotal driver in breast tumor progression leading to transcription of several suites of genes involved in angiogenesis, cell survival, proliferation and an enhanced metastatic phenotype that are advantageous to the neoplastic cells [1]. SIAH2 is part of the ubiquitin ligase complex that target proteins for proteasomal degradation and enhances HIF-1a expression by reducing the abundance of the prolyl hydroxylases [14, 15]. These enzymes in the absence of SIAH2, hydroxylate prolyl residues in the oxygen dependent domain of HIF-1α, resulting in HIF-1α proteosomal degradation and attenutation of the hypoxic response. Since SIAH2 has the potential to profoundly influence the hypoxic response we investigated its expression in normal and neoplastic breast tissues.

We observed significant upregulation of SIAH2 in the nucleus in the transition from normal to *in situ* and invasive carcinomas in breast cancer supporting the notion of an important role of SIAH2 in breast cancer progression. SIAH2 has a nuclear localization signal that could account for its subcellular pattern of expression [16]. The increase in SIAH2 in *in situ* and invasive carcinomas correlates with the hypoxia that occurs in neoplasia as the metabolic demand of the tumor exceed the supply of nutrients and oxygen from the disordered vasculature that is developing.

Correlation analysis showed a significant relationship between high levels of breast tumor SIAH2, negative ER and PR and high HER2. The absence of a positive correlation with ER is of interest since estrogen has been reported to induce expression of SIAH2 in ER-positive breast cancer cell lines [17] and there is a positive relationship between Siah2 and

ER-positive breast tumors but not basal-like phenotype in six public accessible datasets [18-23](analysis not shown). This discrepancy is likely to be due to the well-described differences between gene expression and protein abundance or that ER expression may be heterogeneous in both the pattern and level of expression within tumors. Nevertheless, although there was an inverse relationship between SIAH2 and ER approximately half of SIAH2 positive samples expressed ER suggesting a complex relationship.

The finding that SIAH2 was significantly associated with HER2 and basal-like intrinsic breast cancer subtype, which for basal-like cancers was confirmed in a multivariate analysis, is in accordance with our previous report of an enhanced hypoxic drive in basal-like cancers [24]. In this study, we demonstrate that basal-like breast cancers have an "intrinsically" elevated SIAH2 as part of its phenotype that may partly at least, explain the mechanism underlying high HIF-1α expression in this tumor subtype. The upregulation of SIAH2 may be regulated at several levels. We investigated the potential role p53 may play since this gene is frequently mutated in this tumor type [25]. In support of this notion is the significant correlation between SIAH2 and p53 immunostaining. A further mechanism in basal-like cancer may also involve p38 MAP kinase that is also upregulated in basal-like phenotype as activated p38 increases the activity of SIAH2 [26]. We also explored the role of SIAH2 promoter methylation to assess whether protein expression is epigenetically repressed. We observed no evidence of methylation in any breast cell line, normal breast or in a series of 60 breast cancers of variable phenotype, making this mechanism of repression highly unlikely in breast tissues either in normal or in tumors.

We then hypothesized that since SIAH2 is located on 3q25.1, overexpression might be mediated through gene amplification. Indeed, this locus is frequently amplified in basal-like breast cancer [27] and our preliminary results showed a significant correlation between DNA copy number and mRNA expression, supporting this hypothesis. Specifically we found that basal-like tumors showed copy number gain of the SIAH2 locus more frequently than luminal

tumors and basal-like tumors containing copy number gain were associated with high expression of SAIH2. Nevertheless, using a more sensitive and specific method for quantifying gene copy number such as FISH, together with SIAH2 protein expression in a validation cohort would be of interest to confirm this finding and assess whether true amplification occurs.

Although we observed a significantly shorter relapse-free survival in patients with SIAH2 positive tumors in a univariate analysis, this was not confirmed in the multivariate analysis model that included conventional prognostic factors such as tumor size, grade and lymph node status. Whilst SIAH2 was not an independent survival factor in a multivariante analysis model, it was prognostic in the univariate analysis due to its strong association with the basal-like phenotype, which is an independent prognostic factor. Even so, the role of SIAH2 remains unclear. Thus a report has suggested that SIAH2 positive tumors have a significantly longer progression-free survival in ER positive patients than SIAH2 negative tumors and also that Siah2 levels might be a predictive marker of estrogen responsive disease [28]. The discrepancy between these findings and our own are likely to be due to the use of mRNA levels to measure SIAH2 by Jansen et al [28] and that despite enriching for neoplastic cells, the stromal compartment contributed to the over expression of SIAH2, thus confounding the comparison. In support of this notion, it has been shown by expression microarrays that downregulated SIAH2 in brain metastasis of breast cancer corresponds with low stromal contamination [29]. The concept of SIAH2 being a good prognostic/predictive parameter does not accord with the role of SIAH2 in regulating the hypoxic response or the observation that inhibition of SIAH2 is associated with a reduced metastases in animal models [30]. SIAH2 appears to have several mechanisms of mediating its effect. Some SIAH2 substrates bind directly through an AXVXP motif, some require adaptor proteins, and others are targeted independent of the above sequence motif [3]. Thus, depending on the cell context SIAH2 is likely to have a variety of effects.

SIAH2 is critical to the level of the hypoxic response and therefore is a potential target for anti-cancer therapy. Indeed, since HIF activation results in the regulation of a large number of genes, interference with this pathway would have broad anti-neoplastic effects in contrast to targeting individual genes, such as VEGF with bevacizumab, which is currently used in the clinic. Indeed, menadione, a specific inhibitor of *SIAH2*, increased expression of prolyl hydroxylase with a concomitant decrease in levels of HIF-1α. This promising therapeutic also retarded the growth of melanoma xenografts [31]. The potential of this approach has also been investigated using a short protein fragment that competitively binds to Siah, resulting in reduced breast cancer growth, which appeared to be mediated through inhibition of the hypoxic response [4].

Conclusions

In summary we have shown that *in situ* and invasive breast carcinomas upregulate *SIAH2* and that it is preferentially highly expressed in the basal-like subtype which can be accounted for in part by increased gene copy number. High levels of *SIAH2* may be partly responsible for the enhanced hypoxic drive that underlies this tumor type, which is chemotherapy and radiotherapy resistant. Targeting *SIAH2*, the most apical regulator identified of the hypoxic response pathway, may be a suitable option for anti-cancer therapy in this breast tumor subtype.

Abbreviations:

ER, estrogen receptor; Hif, hypoxia-inducible factor; PR, progesterone receptor; Siah, seven in absentia homologue.

Competing interests:

The authors declare that they have no competing interests.

Authors' contributions

PC carried out the scoring of the TMA and calculated statistics, AM conceived and designed the study, and drafted the manuscript, MCPL and JES stained the TMAs, CSFW assisted staining the TMAs and drafting the MS, NW conducted copy number experiments and analysis, KTH conducted the promoter analysis, AD conducted and supervised promoter analysis, EKAM and SAO generated TMA and scored correlative markers, CMM conducted clinical follow up, RLS provided TMAs, helped drafting the manuscript and provided critical discussions, DB helped designing the study and SBF scored TMAs, conceived and designed the study, and drafted the manuscript. All authors have read and approved the final version of the manuscript.

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Figure legends

Figure 1: Immunohistochemistry of *SIAH2* in normal, in situ and invasive breast carcinomas. (A) Occasional nuclear positivity (arrows) in luminal cells in the terminal duct lobular unit. (B) Moderate to strong staining of *SIAH2* in the nucleus of a small proportion of cell in a high nuclear grade ductal carcinoma in situ with comedo necrosis. (C) Occasional weak to moderate *SIAH2* (arrows) staining in a luminal type ductal carcinoma. (D). Strong *SIAH2* staining in all nuclei in this basal-like breast carcinoma.

Figure 2. Semi-quantitative *SIAH2* expression in normal normal, DCIS and invasive carcinomas samples.

Figure 3: Correlation between *SIAH2* gene copy number changes as assessed by the average logR array value of 8 SNPs which located in the *SIAH2* or within the flanking region of the gene and normalized expression of *SIAH2* in 15 basal-like breast cancers.

Figure 4. Kaplan Meier curves stratified by *SIAH2* expression for relapse-free survival in (A) all tumors (n = 246) (B) Luminal tumors (n=144).

Table 1. **Chi square tests, *SIAH2* expression in normal breast, DCIS and invasive cancers (p<0.0001)**

Tissue type	negative	positive	Total
Normal	10(100%)	0(0%)	10(100%)
DCIS	47(87%)	7(13%)	54(100%)
Invasive	155 (45%)	194 (55%)	349(100%)
Total	**212(51%)**	**201 (49%)**	**413 (100%)**

DCIS, Ductal Carcinoma in situ

Table 2. Contingency table of SIAH2 expression in invasive breast carcinomas of the combined cohort with clinicopathological parameters

	negative 169 (45%)	positive 209 (55%)	Total 378 (100%)	P value
Grade				**< 0.0001**
Low	40 (78.4%)	11 (21.6%)	51 (100%)	
Intermediate	77 (60.2%)	51 (39.8%)	128 (100%)	
High	32 (20.4%)	125 (79.6%)	157 (100%)	
Age				**0.187**
<50	48 (39.3%)	74 (60.7%)	122 (100%)	
>50	107 (47.8%)	117 (52.1%)	224 (100%)	
Size				**0.168**
<20 mm	89 (48.6%)	94 (51.4%)	183 (100%)	
>20 mm	61 (39.9%)	92 (60.1%)	153 (100%)	
Lymph node				**0.915**
Negative	83 (45.4%)	100 (54.6%)	147 (100%)	
Positive	64 (43.0%)	85 (57.0%)	185 (100%)	
ER				**< 0.0001**
Negative	21 (20.8%)	80 (79.2%)	101 (100%)	
Positive	129 (54.4%)	108 (45.6%)	237 (100%)	
PR				**< 0.0001**
Negative	39 (28.5%)	98 (71.5%)	137 (100%)	
Positive	111 (55.2%)	90 (44.8%)	201 (100%)	
HER2				**0.003**
Negative	128 (48.3%)	137 (51.7%)	265 (100%)	
Positive	22 (29.7%)	52 (70.3%)	74 (100%)	
Subtype				**< 0.0001**
Luminal	114 (57.6%)	84 (42.4%)	198 (100%)	
Basal-like	4 (9.6%)	37 (90.2%)	41 (100%)	
Her2	22 (29.3%)	53 (70.7%)	75 (100%)	
Null	11 (44%)	14 (56%)	25 (100%)	
P53				
Negative	108 (57.8%)	79 (42.2%)	187 (100%)	**< 0.001**
Positive	12 (18.2%)	54 (81.8%)	66 (100%)	

ER, estrogen receptor; PR, progesterone receptor.

Table 3. Multivariate analysis in the combined cohort (n=378) using binary logistic regression model of the effect of *SIAH2* expression on tumor subtype (with luminal tumors as a reference), grade, lymph node status and size

	P value	*Hazard Ratio*	*95% C.I. for hazard ratios*
Tumor type			
Luminal (ref)	0.09		
Basal	0.02	4.04	1.3-12.8
HER2	0.15	1.64	0.8-3.2
Null	0.67	1.23	0.5-3.2
Grade			
1	0.000		
2	0.02	2.61	1.2-5.8
3	0.0001	12.72	5.2-31.4
Lymph node status	0.28	0.75	0.4-1.3
Size > 20mm	0.18	0.69	0.4-1.2

Table 4. Multivariate analysis, Cox regression model of relapse-free survival in all breast cancers of validation cohort (n=245)

	P value	*Hazard Ratio*	*95% C.I. for hazard ratios*
SIAH2	0.47	1.24	0.7-2.2
Grade			
1 (reference)	0.54		
2	0.70	0.83	0.3-2.1
3	0.70	1.22	0.4-3.3
Size	0.12	1.37	0.9-2.2
Lymph node status	0.001	2.25	1.4-3.7
Tumor type			
Luminal	0.004		
Basal-like	0.005	3.0	1.4-6.4
HER2	0.001	2.9	1.6-5.7
Null	0.016	2.5	1.2-5.1

Additional files:

Additional file 1:

Supplementary Table 1:

Flow of breast cancer patients through the study, according to REMARK criteria (24).

Additional file 2:

Supplementary Table 2:

Tumour phenotype of 60 samples for which SIAH2 methylation analysis was performed.

Additional file 3:

Supplementary Table 3:

Contingency table of DCIS and available clinicopathological variables.

Additional file 4:

Supplementary Table 4:

Contingency table of SIAH2 expression in invasive breast carcinomas of the primary cohort with clinicopathological parameters.

Additional file 5:

Supplementary Table 5:

Contingency table of SIAH2 expression in invasive breast carcinomas of the initial cohort with clinicopathological parameters.

Additional file 6:

Supplementary Figure 1:

SIAH2 methylation in breast carcinoma samples: MS-HRM detects sample methylation status by melting the amplicons after PCR. Methylated samples melt later than unmethylated samples as they have cytosines in their sequences rather than the thymines after the bisulfite modification. Two ER positive and two ER negative breast carcinomas showing no methylation in *SIAH2*. Standard controls of 100%, 50%, 10% and 0% methylation are shown. The curve for each sample represents data from duplicate samples.

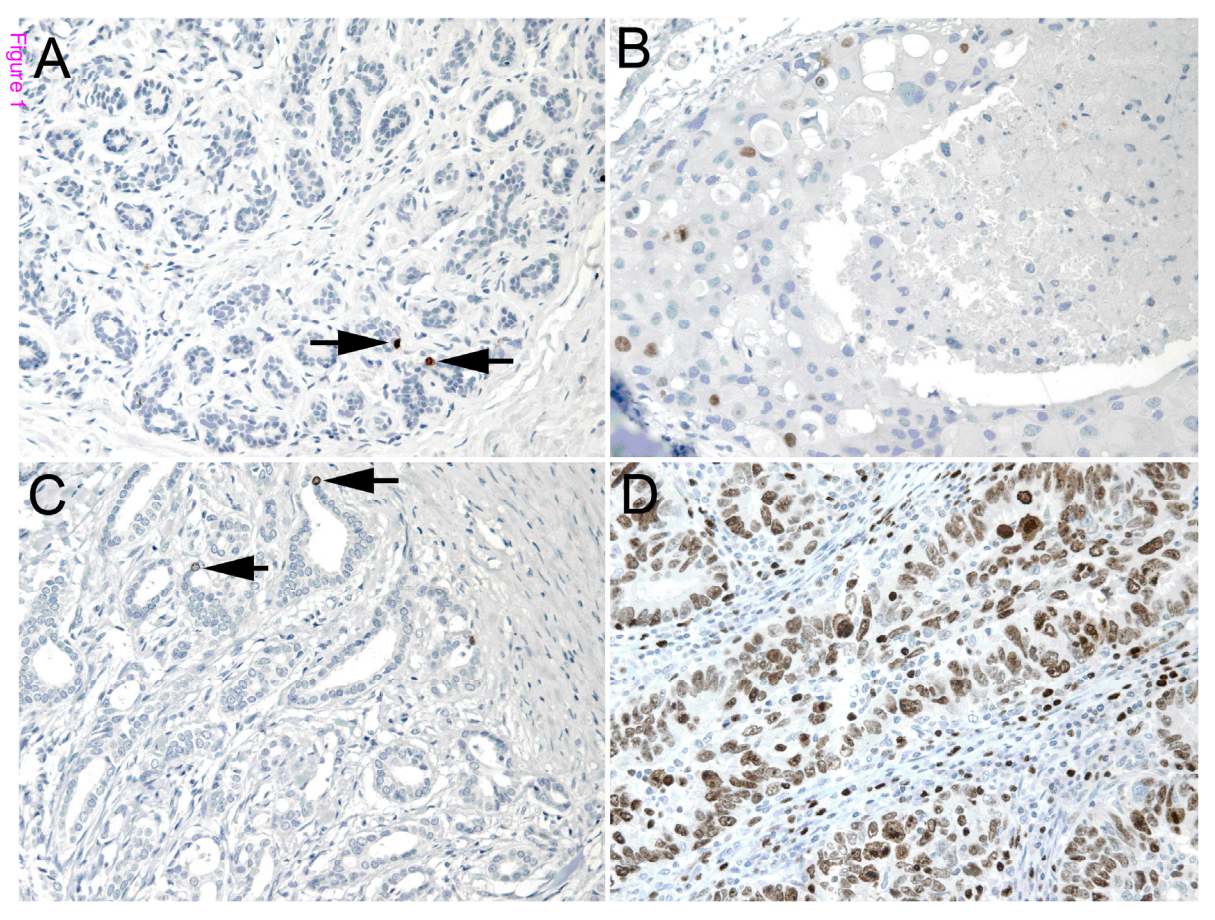


Figure 1
A
B
C
D

Figure 2

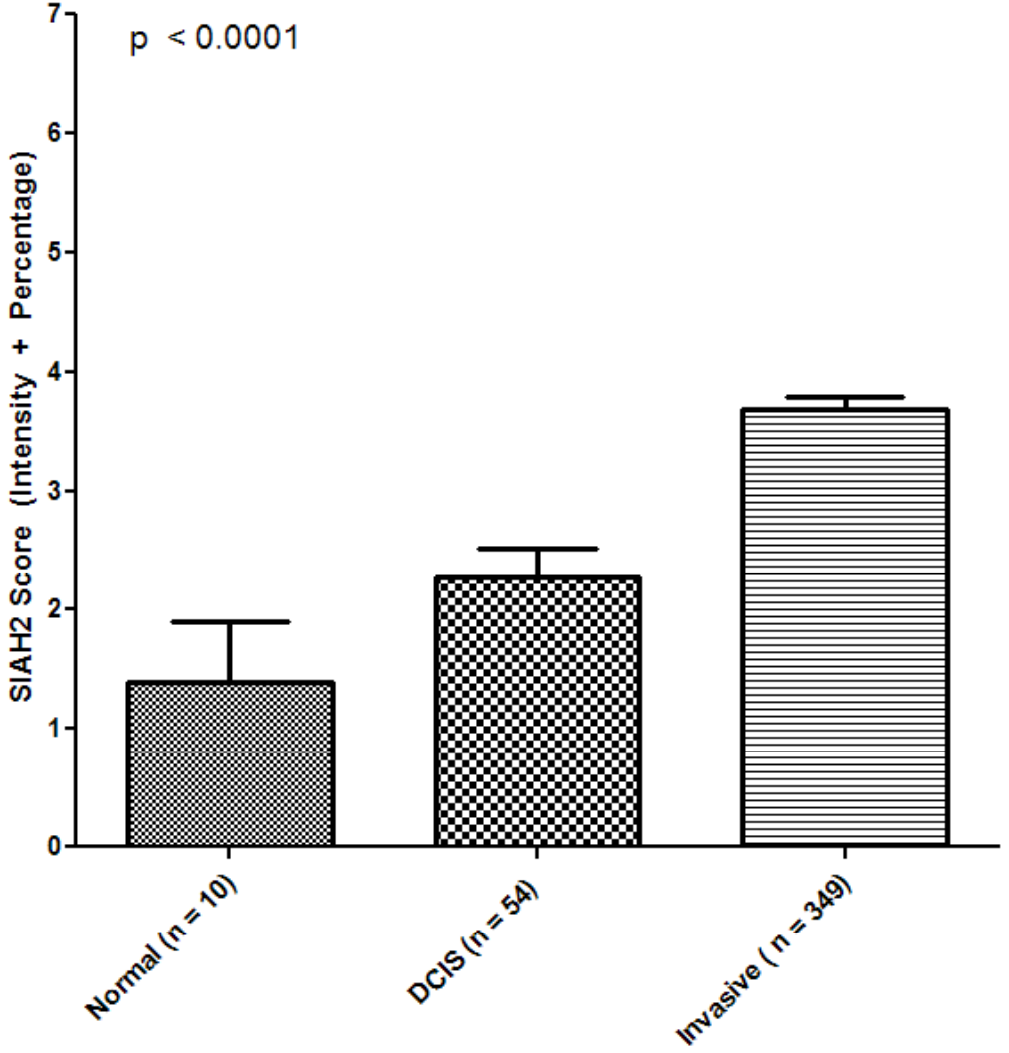

p < 0.0001
SIAH2 Score (Intensity + Percentage)
0
1
2
3
4
5
6
7
Normal (n = 10)
DCIS (n = 54)
Invasive (n = 349)

Figure 3

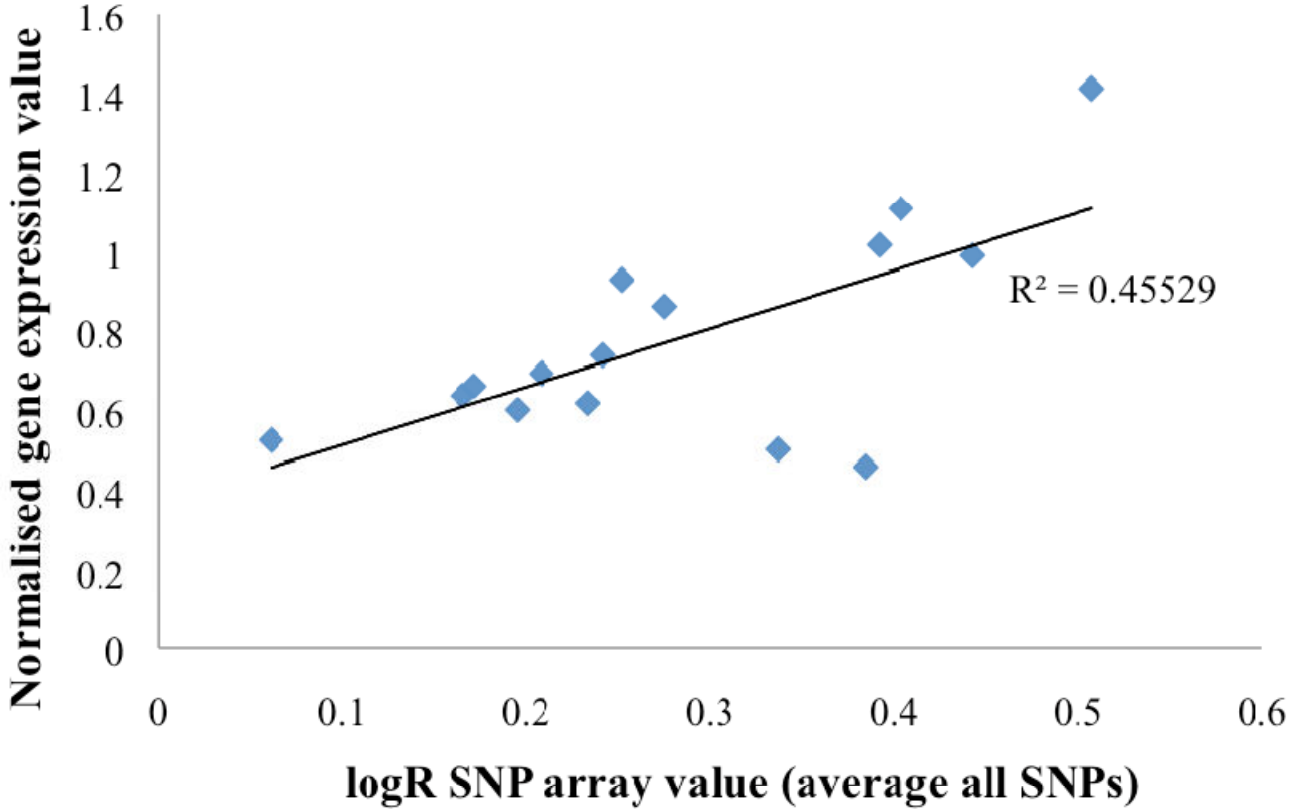
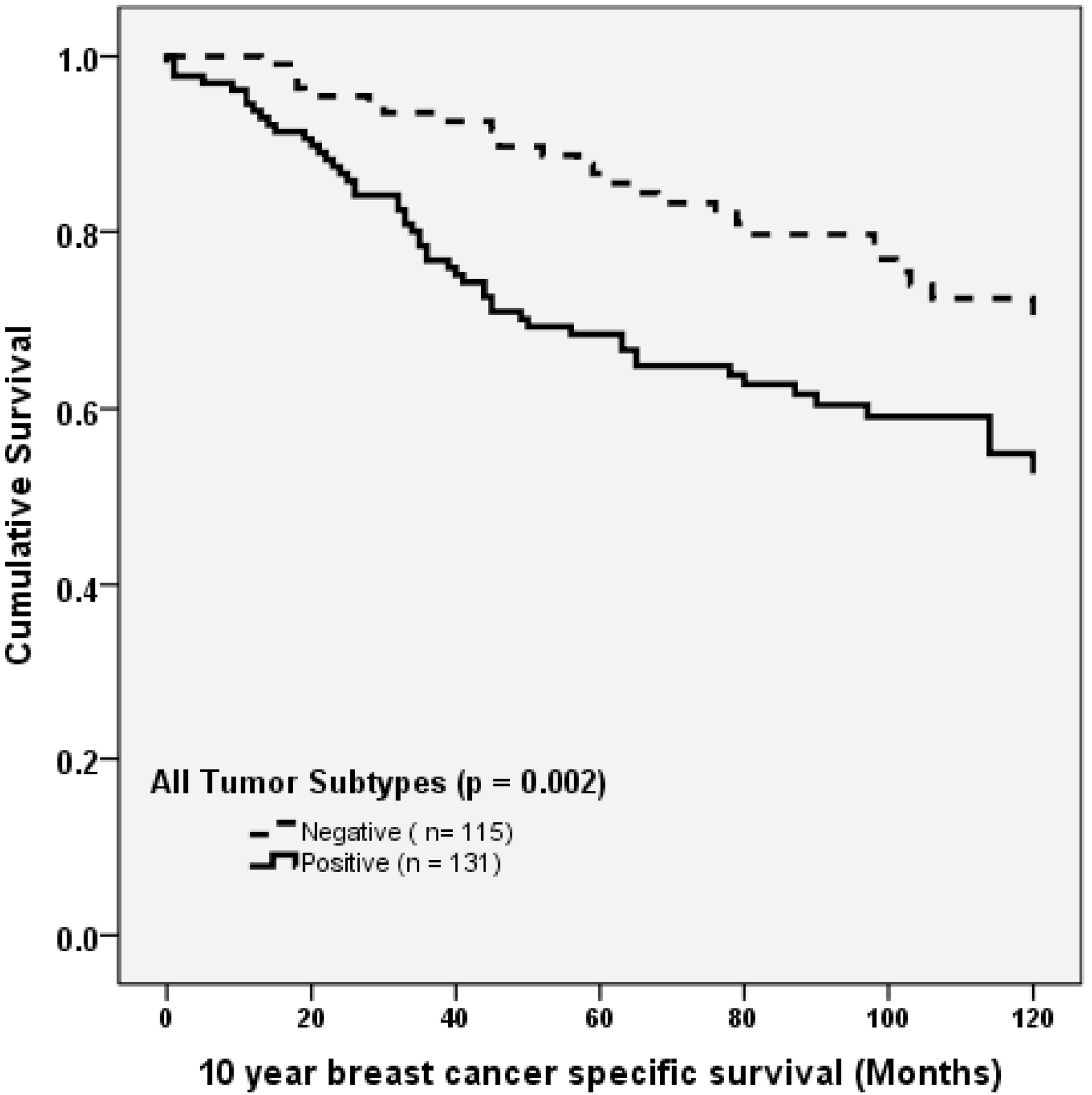

Normalised gene expression value
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
R² = 0.45529
0
0.1
0.2
0.3
0.4
0.5
0.6
logR SNP array value (average all SNPs)

Figure 4


Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
All Tumor Subtypes (p = 0.002)
Negative (n= 115)
Positive (n = 131)
0
20
40
60
80
100
120
10 year breast cancer specific survival (Months)

Additional files provided with this submission:

Additional file 1: Supplementary Table 1.ppt, 62K
http://breast-cancer-research.com/imedia/1536816076516010/supp1.ppt
Additional file 2: Supplementary Table 2.ppt, 29K
http://breast-cancer-research.com/imedia/2461612715160106/supp2.ppt
Additional file 3: Supplementary Table 3.ppt, 66K
http://breast-cancer-research.com/imedia/689376795160107/supp3.ppt
Additional file 4: Supplementary Table 4.ppt, 72K
http://breast-cancer-research.com/imedia/1890012285160107/supp4.ppt
Additional file 5: Supplementary Table 5.ppt, 70K
http://breast-cancer-research.com/imedia/1989572298516010/supp5.ppt
Additional file 6: Supplementary Figure1.ppt, 71K
http://breast-cancer-research.com/imedia/1722223513516010/supp6.ppt