The prognostic significance of Heat Shock Protein 90AA1 (HSP90α) in invasive breast cancer

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ABSTRACT

Aims: The mechanisms that drive breast cancer (BC) progression and poor outcome are not fully understood. The human heat shock protein 90 alpha family class A member 1 (HSP90α) encoded by the HSP90AA1 gene has a vital role in cellular responses to stress and is implicated in the development and progression of many cancers. The current study aims to explore the clinical and prognostic importance of HSP90α in BC.

Methods: The Molecular Taxonomy of Breast Cancer International Consortium (n=1980); The Cancer Genome Atlas (n=1097) and the Breast Cancer Gene-Expression Miner (Bc-GenExMiner) BC datasets (n=5056) were used to evaluate HSP90AA1 mRNA expression. HSP90α protein expression was further assessed using immunohistochemistry in a large (n=911) well-characterised BC series. The association between mRNA and protein expressions with other clinicopathological parameters and outcome was analysed.

Results: High expression of HSP90AA1 both at the mRNA and protein levels was significantly associated with characteristics of BC poor prognosis, including high grade, lymphovascular invasion, poor Nottingham Prognostic Index and positive expression of p53 and PIK3CA. Outcome analysis revealed that high HSP90α protein expression is an independent predictor of shorter BC-specific survival.

Conclusion: HSP90α can be used as a potential prognostic marker in BC. Further mechanistic studies are warranted to determine the underlying molecular mechanisms mediated by HSP90α in BC.

INTRODUCTION

Breast Cancer (BC) is a heterogeneous group of diseases with various presentations, morphological features, outcome and response to therapy (1). Although many factors that influence BC incidence and progression have been identified, current understanding of the diverse molecular and cellular mechanisms contributing to BC progression remains limited. A better knowledge of the factors involved in BC progression will help in tailoring personalised therapy and improving outcome (2).

Existing data indicate that the cellular stress response is important in cancer progression and cancer therapy (3). Expression of Heat Shock Proteins (HSPs) increases in response to cellular stress (4, 5) and can enable tumour cells to survive cellular stress by evading cell death and senescence mechanisms (6). Consistent with this, it was demonstrated that HSPs have an essential role in multiple pro-oncogenic signalling pathways, and it is an Adenosine Triphosphate (ATP) dependent protein chaperone (7, 8). Subsequently, the heat shock protein 90 (HSP90) family has attracted considerable attention as a potential therapeutic target (9) as these proteins are the key players involved in protein modification and have been implicated in BC progression (10). The human HSP90 alpha family class A member 1 (HSP90α), encoded by *the HSP90AA1* gene, appears to be critically important in the prevention of cell death (11) and evasion of apoptosis (12). Previous studies have indicated that HSP90α overexpression inhibits major apoptosis associated proteins in leukaemia, enabling the malignant cells to escape apoptosis and undergo proliferation (13, 14). Consistent with this, mechanistic evidence from a lung cancer model indicates HSP90α role in tumour growth (15).

Autophagy can induce genomic instability, malignant transformations and may help metastatic cancer cells to escape from anoikis (16, 17). *In vitro* studies in osteosarcoma have also shown that *HSP90AA1* knockdown increased chemo-sensitivity by decreasing autophagy activity of cancer cells (18), which raises the potential that HSP90α also contributes to pro-invasive

mechanisms. Taken together, this study aimed to investigate the clinical and prognostic value of HSP90α expression, at both mRNA and protein levels, in BC and relating these findings to key clinicopathological factors and the expression of other proteins relevant to HSP90α function.

MATERIAL AND METHODS

HSP90AA1 mRNA expression

The clinical significance of *HSP90AA1* mRNA expression was assessed using the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset of invasive BC (n=1980) (19, 20). The clinicopathological associations and prognostic value of *HSP90AA1* mRNA expression were further evaluated using BC cohort of The Cancer Genome Atlas (TCGA)- Breast Invasive Carcinoma BRCA (n=1097) (21) and the Breast Cancer Gene-Expression Miner v4.5 (Bc-GenExMiner v4.5) datasets (n= 5,056) (22).

HSP90α protein expression

This study included a well-characterised cohort of invasive BC (n=911) collected from the Nottingham City Hospital, Nottingham, UK. Treatment data and consequent outcome were maintained and updated. The outcome data included breast cancer-specific survival (BCSS) and distant metastasis-free survival (DMFS). The BCSS was defined as duration (in months) from primary surgical treatment until the time of death due to BC. DMFS refers to the duration (in months) from primary surgical treatment until the first distant metastasis (23). Nottingham Prognostic Index (NPI) was used to risk stratify and manage patients (24), where the good prognostic group (NPI ≤3.4) received no therapy while all patients with ER-positive tumours and had moderate and poor NPI (>3.4) received adjuvant hormonal therapy (HT). The poor prognostic NPI group of ER-negative patients were offered chemotherapy if fit. HT coupled

with chemotherapy was given to patients with both ER and lymph node (LN) positive patients (25). A total of 330 patients (36%) received HT, and 165 (18%) were given chemotherapy. Patients` characteristics of the 911 cases are summarised in Supplementary Table 1.

Immunohistochemistry (IHC)

To validate the antibody-specificity, western blotting was performed using the anti-HSP90α antibody (ab13492; Abcam, Cambridge, UK) with a dilution of 1:700. The house-keeping rabbit monoclonal anti-β-Actin (ab13492; Abcam, Cambridge, UK) was used at a dilution of 1:5000. For western blotting, cell lysates from HeLa, MCF7, SKBr3 and MDA-MB-231 (all from The American Type Culture Collection; Rockville, MD, USA) were used. A single specific band at the predicted molecular weight (90 kDa; Supplementary Fig.1a) confirmed the specificity of the antibody. Full-face BC tissue sections (n=14) that were selected based on lymphovascular invasion (LVI) status, tumour grades, and histological type, were stained using IHC to evaluate the pattern of HSP90α protein expression before staining of Tissue Microarrays (TMAs). TMA was constructed from the study cohort, as previously described (26). HSP90α protein expression was assessed by IHC using the Novolink Max Polymer Detection system (Leica, Newcastle, UK). Anti-HSP90α primary antibody, diluted at 1:200, was incubated for 1 hour at room temperature.

Scoring of protein expression

HSP90α stained slides were scanned into high-resolution digital images using a high throughput digital scanner (NanoZoomer, Hamamatsu Photonics) (27). The images were viewed at 20x magnification using Xplore image viewer software (Philips, UK). Only cores that contained adequate tumour tissue (at least 15% of the invasive tumour relative to the whole core area) were scored. About one-third of cases were excluded due to loss of cores (during

TMA sectioning, processing and/or during IHC staining process) or presence an insufficient tumour tissue (<15% of the core surface area) for assessment. The modified 'H-Score' method was used semiquantitative evaluation taking the percentage (0-100) and the intensity (0 - negative, 1 -weak, 2 -moderate and 3 -strong) of stained cells into consideration (28). For interobserver concordance, a subset of cores (25%) was independently scored by another scorer, (MT) and interclass correlation coefficient (ICC) was performed (ICC=0.7). Scoring was performed on anonymised samples of clinical variables at the time of scoring. Discordant cases were re-scored by both observers, and a final score was agreed.

BC molecular subtypes were defined according to their IHC profile as Luminal-A: ER+, PR+/HER2-, Luminal-B (ER+/HER2- with high proliferation (Ki67≥10%) or ER+/HER2+ regardless the proliferation index), HER2-enriched (HER2+ and ER-, PR-), and Triple-negative breast cancer (TNBC) (ER-, PR- and HER2-). Data on biomarkers related to EMT, progression and metastasis of BC, including N-cadherin, Ki67, PIK3CK kinase, tumour suppressor protein p53, AKT and epidermal growth factor receptor was available and used in this study. Details of these markers' expression and cut off values were described in previous publications (28-34).

Statistical analysis

SPSS 24 (IBM, Chicago, IL, USA) was used to perform the statistical analysis. For *HSP90AA1* mRNA expression, the median value was used to dichotomise the data. For the HSP90α protein expression, the cut-off point was determined as H-score 50 using the median, with a score >50 was considered high expression. The association between HSP90α expression and the clinicopathological factors was evaluated using the Chi-square test. The univariate survival analysis was conducted using Kaplan Meier plots, and multivariate survival analyses were obtained using Cox regression model with adjustment of covariates was fitted to test

independence from standard prognostic factors in BC. A $p < 0.05$ (two-tailed) was considered significant. Correlation between *HSP90AA1* mRNA and protein expression was analysed with Spearman's coefficient correlation test, using the subset of the Nottingham series included in METABRIC study (n=153).

This study was approved by the Nottingham Research Ethics Committee 2 (under the title: Development of molecular genetic classification of BC) reference number REC202313. This study was conducted in accordance with the Declaration of Helsinki, and informed consent was obtained from all patients who participated in the study.

RESULTS

HSP90AA1 mRNA expression

High expression of *HSP90AA1* mRNA, as defined using median cut off, was seen in (990 of 1980 cases) in METABRIC. A similar frequency of positivity was seen in the TCGA-BRCA (549 of 1097 cases) and the Bc-GenExMiner v4.5 datasets (2537 of 5056 cases). High expression of *HSP90AA1* mRNA was significantly associated with high grade, LVI-positivity, ER-negativity and HER2-positivity (Table 1a). *HSP90AA1* mRNA expression was significantly higher in luminal B, HER2-positive and TNBC than luminal A tumours (Table 1a), and it was associated with high PIK3CA and p53 (Table 1b). Previously stated data was seen in both METABRIC and TCGA-BRCA.

Outcome analysis revealed that patients who had BC with higher *HSP90AA1* mRNA expression had significantly shorter BCSS than those with low *HSP90AA1* expression (HR=1.38; 95% CI=1.16-1.65, $p = 0.0004$; and HR=1.85; 95% CI=1.318-2.61, $p<0.0001$ in METABRIC and TCGA data respectively; Fig.1a and b). Using the prognostic module of Bc-GenExMiner v4.5, *HSP90AA1* mRNA was validated as a significant prognostic factor for shorter survival (HR=1.17; 95% CI=1.07-1.28, $p = 0.0004$; Supplementary Fig.1b).

HSP90α protein expression and its association with the clinicopathological parameters

HSP90α staining was homogeneously distributed within the invasive tumour cells in the full-face tissue sections. HSP90α expression in invasive BC and adjacent normal terminal duct-lobular units (TDLUs) are illustrated in (Fig.2a-b). HSP90α showed cytoplasmic immunoreactivity and a positive linear correlation with *HSP90AA1* mRNA (Spearman's coefficient 0.3; p=0.001). The normal TDLUs adjacent to the tumour showed uniformly weak HSP90α staining compared to invasive cancer cells.

High HSP90α protein expression was observed in 58% of cases and was significantly associated with high tumour grade ($p < 0.0001$), positive nodal status ($p = 0.0016$), positive LVI ($p = 0.014$), high PIK3CA ($p < 0.0001$) and positive p53 ($p < 0.002$), Table 2. HSP90α was highly expressed in Luminal-B / HER2-negative, TNBC, Luminal-B / HER2-positive and HER2-enriched subtypes while Luminal-A tumours showed the lowest levels of expression (p=0.002), Table 2.

Outcome analysis

Cases with high HSP90α protein expression had significantly shorter BCSS than those with low HSP90α expression (HR=1.45; 95% CI=1.17-1.89, $p = 0.005$; Fig.3a). In addition, cases with high HSP90α expression displayed significantly shorter DMFS (HR= 1.35; 95% CI 1.65-1.74, $p = 0.013$; Fig.3b). High expression of HSP90α in Luminal-B / HER2-negative cohort was associated with shorter BCSS and DMFS (p = 0.002, p = 0.009, respectively; Supplementary Fig.2a-b). There was a similar trend observed in TNBC cases, while there was no association with outcome in the other molecular classes. Multivariate Cox regression analysis for HSP90α, adjusting for standard prognostic parameter including tumour size, nodal stage and LVI, showed that high HSP90α protein expression was an independent poor prognostic factor for shorter BCSS (HR=1.38; 95% CI=1.06-1.80, $p = 0.018$), and DMFS (HR=1.32; 95% CI=1.03-1.69, $p = 0.027$; Supplementary Table 2).

DISCUSSION

BC is a group of heterogeneous diseases that differ in morphological, molecular, and clinical behaviour. This heterogeneity poses challenges in understanding the biology and the mechanisms underlying BC progression (2). BC risk stratification and prediction of the usefulness of different therapeutic protocols currently depend on the tumour clinicopathological criteria in addition to clinically validated biomarkers such as ER, PR and HER2. However, substantial proportions of patients with apparently similar clinicopathological features can show variable outcomes and different responses to the same therapeutic regimens. HSP family of proteins have essential roles in multiple oncogenic signalling pathways, through acting to maintain cellular proteins in their correct configurations to ensure their stability (7, 8). HSP90α controls protein assembly with proven extracellular roles in wound healing and cancer (35, 36). Therefore, the functions of HSP90α confer a favourable mechanism used by cancer cells to facilitate cancer cell migration and metastasis (37, 38). Thus, we explored the expression of HSP90α in multiple BC datasets at mRNA and protein level to correlate with clinicopathological parameters and prognostic utility. The current study shows that high expression of HSP90α is associated with poor prognostic parameters and an independent prognostic marker of poor clinical outcome.

Our results showed that high expression of *HSP90AA1* mRNA was significantly associated with clinicopathological variable characteristics of poor prognosis, including high histological grade, higher NPI prognostic scores, and loss of hormone receptor expression. Importantly, the expression was associated with shorter patients' survival, independent of other variables. These results are in line with the findings from a recent study investigating the mRNA expression of six HSPs in BC and reported an association between *HSP90AA1* and poor prognosis (6).

Patients with non-small cell lung cancer and lung adenocarcinoma showed that high mRNA expression of *HSP90AA1* is associated with poor outcome (39).

The positive correlation of HSP90α with PIK3CA and p53 expression, (40, 41) indicates that high HSP90α expression is associated with aggressive BC. PIK3CA is an EMT-promoting protein that contributes to the cell cycle control and interacts with HSP90 leading to apoptosis inhibition (4, 42). During the cell cycle, the active form of PIK3CA can phosphorylate the regular component of the membrane PIP2 to PIP3 which in turn activates AKT protein kinase B that interacts with HSP90 leading to inhibition of apoptosis. *In vivo* studies have reported the increased sensitivity of the cells to an apoptosis inducing stimulus by inhibiting AKT-HSP90 binding (43, 44). These results were supported by another study that showed HSP90 inhibition enhances AKT dephosphorylation process (45). I*n vitro* and *in vivo* experiments have also concluded that PI3K/AKT is a major contributing signalling pathway in Burkitt lymphoma (40). Wild type p53 is well known as a tumour suppressor, whereas mutant p53 seems to acquire oncogenic phenomenon (46, 47). Interestingly, the activation of HSP90 expression induces stabilisation of mutant p53, consequently promoting tumour progression and metastases through the Hsp90-p53 co-binding mechanism (41, 48).

Lymph node stage and LVI in BC are strong prognostic factors (49) predicting patient outcome. In our study, HSP90AA1 mRNA and protein expression was strongly associated with LVI positivity and positive nodal stage. Luminal B, TNBC and HER2 enriched BC molecular subtypes are biologically and clinically aggressive subtypes compared to luminal A tumours (50-52). Our results showed that HSP90α is less expressed in the Luminal-A BC molecular subtype than the Luminal-B / HER2-negative, Luminal-B / HER2-positive, HER2 enriched and TNBC classes. This could reflect the potential interaction between HSP90α expression,

tumour proliferation, and HER2 signalling pathway, which may trigger invasion and LVI resulting in BC metastasis (53). In line with our findings, clinical trials of HSP90 inhibitors have shown promising results in the Luminal B and HER2-positive tumours (54).

In conclusion, the current results highlighted the significant association of HSP90α with poor prognosis in BC patients and especially in the intrinsic subtypes of aggressive behaviour including Luminal B. Functional studies are required to determine the influence of aberrant expression of HSP90α and to explore its utility as a potential therapeutic target for precision medicine.

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Compliance with ethical standards

Funding: Funding and support of the current study were made possible through the Saudi Arabia Ministry of Education, Northern Borders University (NBU).

Research involving human participants and/or animals: The researchers of the current study did not perform any experiment on either human or animal subjects.

Competing Interest: All the researchers involved in the current study declare no competing of interest in conducting this research

Informed Consent: The present study acquired the ethics approval of the North West – Greater Manchester Central Research Ethics Committee to use human tissue samples from the Nottingham Health Science Biobank (NHSB), along with the reference number 15/NW/0685. Before surgery, the informed consent was retrieved from all the study patients, informing them that their tissue materials will be used in research. The study followed the REMARK guidelines for tumour marker prognostic studies.

Availability of data and materials: The researchers validate that the information used in the current study is available upon request.

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Table 1a Association between *HSP90AA1* mRNA expression and clinicopathological characteristics in the METABRIC and TCGA invasive breast cancer datasets

		METABRIC cohort			**TCGA cohort**		
Parameters		**Expression of *HSP90AA1***			**Expression of *HSP90AA1***		
		Low No (%)	**High No (%)**	**p-value**	**Low No (%)**	**High No (%)**	**p-value**
Tumour size	<2cm	451 (52.6)	407 (47.4)	**0.041**	99 (55.9)	78 (44.1)	**0.01**
	≥ 2cm	528 (47.9)	574 (52.1)		273 (45.7)	325 (54.3)	
Histological grade	Grade 1,2	534 (56.8)	406 (43.2)	**< 0.0001**	158 (59.0)	110 (41.0)	**< 0.0001**
	Grade 3	405 (42.5)	547 (57.5)		67 (33.7)	132 (66.3)	
Nodal status	Negative	542 (52.4)	493 (47.6)	**0.026**	423 (47.0)	477 (53.0)	0.564
	Positive	444 (47.3)	494 (52.7)		10 (47.6)	11 (52.4)	
Lymphovascular invasion	Negative	505 (54.3)	425 (45.7)	**0.017**	324 (58.0)	235 (42.0)	**< 0.0001**
	Positive	306 (48.2)	329 (51.8)		127 (43.1)	168 (56.9)	
Oestrogen receptor status	Negative	215 (45.4)	259 (54.6)	**0.02**	100 (42.0)	138 (58.0)	**0.001**
	Positive	775 (51.5)	731 (48.5)		431 (53.5)	375 (46.5)	
Progesterone receptor status	Negative	459 (48.8)	481 (51.2)	0.32	148 (43.0)	196 (57.0)	**< 0.0001**
	Positive	531 (51.1)	509 (48.9)		380 (54.5)	317 (45.5)	
HER2	Negative	897 (51.8)	836 (48.2)	**< 0.0001**	311 (53.2)	274 (46.8)	**< 0.0001**
	Positive	93 (37.7)	154 (62.3)		61 (34.5)	116 (65.5)	
Nottingham prognostic index	GPG	390 (57.4)	290 (42.6)	**< 0.0001**			
	MPG	510 (46.3)	591 (53.7)				
	PPG	90 (45.2)	109 (54.8)				
Molecular subtypes	Luminal A	430 (59.9)	288 (40.1)	**< 0.0001**	301 (60.3)	198 (39.7)	**< 0.0001**
	Luminal B	169 (34.6)	319 (65.4)		59 (29.9)	138 (70.1)	
	HER2 enriched	105 (43.8)	135 (56.3)		16 (20.5)	62 (79.5)	
	Basal-like	148 (45.0)	181 (55.0)		74 (43.3)	97 (56.7)	
	Normal-like	134 (67.3)	65 (32.7)		30 (83.3)	6 (16.7)	

Values in bold are statistically significant.

GPG, good prognostic group; METABRIC, The Molecular Taxonomy of Breast Cancer International Consortium; MPG, moderate prognostic group; PPG, poor prognostic group; TCGA, The Cancer Genome Atlas.

Table 1b Association between *HSP90AA1* mRNA expression and other Gene of Interest in the METABRIC and TCGA invasive breast cancer datasets

Gene of Interest	***HSP90AA1*mRNA Level (METABRIC)**		**Mean Rank**		***Z*-score**	***p*-value**	***HSP90AA1*mRNA Level (TCGA)**		**Mean Rank**		***Z*-score**	***p*-value**
	Low (%)	**High (%)**	**Low**	**High**			**Low (%)**	**High (%)**	**Low**	**High**		
Ki67	990 (50)	990 (50)	920	**1060**	-5.4	**<0.001**	548(49.9)	549 (50)	443	**654**	-11	**<0.001**
N-Cadherin			967	1013	-1.78	0.075			506	591	-4.4	<0.001
PIK3CA			982	**998**	-3.15	**0.002**			483	**614**	-6.9	**<0.001**
P53			941	**1039**	-3.8	**<0.001**			550	**547**	-0.1	0.889
AKT			981	999	-0.72	0.471			506	591	-4.4	**<0.001**
EGFR			1011	969	-1.66	0.096			554	543	-0.6	0.558

Values in bold are statistically significant.

EGFR, Epidermal Growth Factor Receptor; METABRIC, The Molecular Taxonomy of Breast Cancer International Consortium; PIK3CA, Phosphoinositide-3-kinase, Catalytic, Alpha polypeptide; TCGA, The Cancer Genome Atlas.

Table 2 Associations between HSP90α protein expression and the clinicopathological factors

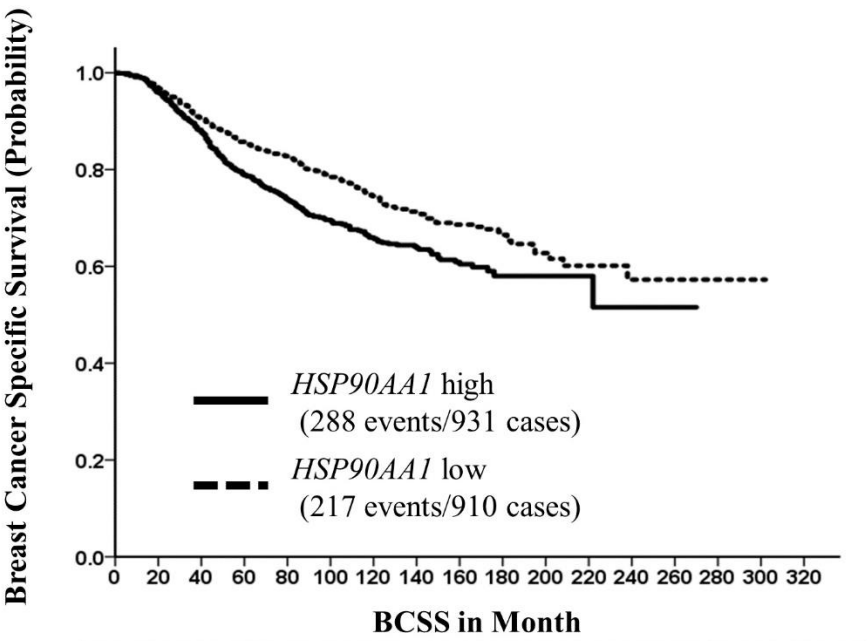
Factors		Expression of HSP90α		
		Low N (%)	**High N (%)**	**p-value**
Tumour size	≤ 2cm	190 (43.9)	243 (56.1)	0.435
	> 2cm	195 (41.3)	277 (58.7)	
Histological grade	Grade 1,2	234 (50.6)	228 (49.4)	**< 0.0001**
	Grade 3	150 (34.1)	290 (65.9)	
Nodal status	Negative	255 (45.6)	304 (54.4)	**0.016**
	Positive	130 (37.5)	217 (62.5)	
Lymphovascular invasion	Negative	271 (45.5)	325 (54.5)	**0.014**
	Positive	114 (37.0)	194 (63.0)	
Oestrogen receptor status	Negative	85 (37.8)	140 (62.2)	0.126
	Positive	296 (43.6)	383 (56.4)	
Progesterone receptor status	Negative	144 (39.9)	217 (60.1)	0.534
	Positive	215 (42.0)	297 (58.0)	
Nottingham prognostic index	GPG	149 (53.8)	128 (46.2)	**< 0.0001**
	MPG	182 (38.6)	290 (61.4)	
	PPG	51 (34.5)	97 (65.5)	
Molecular subtypes	Luminal A	115 (54.0)	98 (46.0)	**0.002**
	Luminal B/HER2-	98 (36.3)	172 (63.7)	
	Luminal B/HER2+	16 (39.0)	25 (61.0)	
	Triple Negative	57 (36.5)	99 (63.5)	
	HER2 enriched	37 (42.0)	51 (58.0)	
Ki67	Negative	107 (41.6)	150 (58.4)	0.352
	Positive	163 (45.4)	196 (54.6)	
N-cadherin	Negative	80 (43.5)	104 (56.5)	0.35
	Positive	190 (39.5)	291 (60.5)	
PIK3CA	Negative	95 (55.2)	77 (44.8)	**< 0.0001**
	Positive	191 (36.1)	338 (63.9)	
P53	Negative	278 (44.8)	342 (55.2)	**0.002**
	Positive	83 (33.6)	164 (66.4)	
AKT	Negative	59 (39.9)	89 (60.1	0.861
	Positive	182 (39.1)	284 (60.9)	
EGFR	Negative	300 (43.2)	395 (56.8)	0.083
	Positive	66 (36.1)	117 (63.9)	

Values in bold are statistically significant.
EGFR, Epidermal Growth Factor Receptor; GPG, good prognostic group; MPG, moderate prognostic group; PIK3CA, Phosphoinositide-3-kinase, Catalytic, Alpha polypeptide; PPG, poor prognostic group

Figure Legends

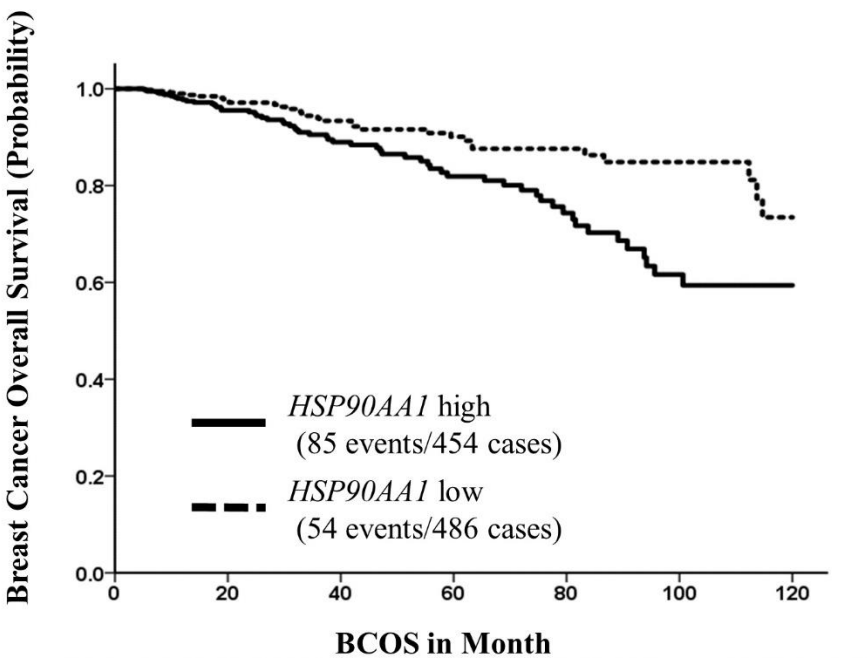
Figure. 1

a)



Hazard Ratio: 1.38; 95% CI: 1.16-1.65, p=0.0004

b)

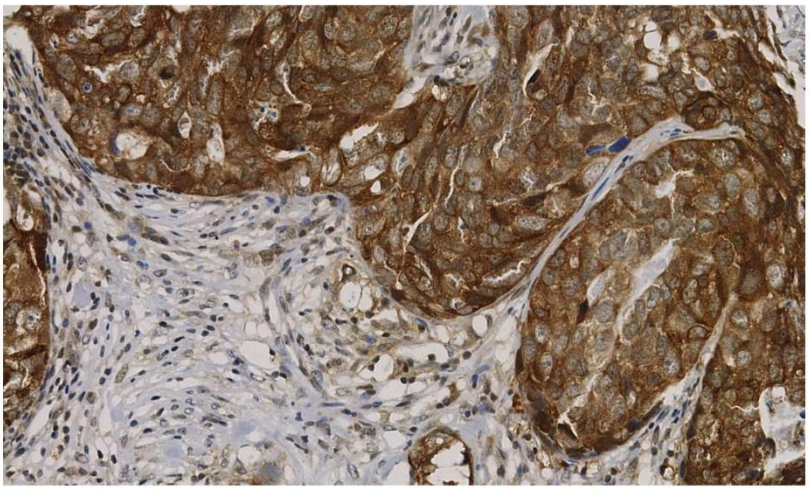


Hazard Ratio: 1.85; 95% CI: 1.318-2.61, p<0.0001

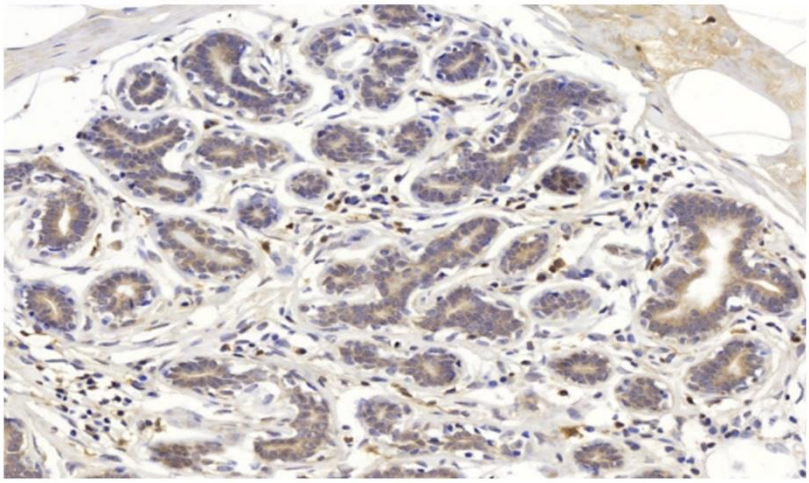
Fig. 1: Cumulative survival of BC patients stratified by *HSP90AA1* mRNA expression. Breast Cancer Specific Survival in the METABRIC cases was significantly worse in the high *HSP90AA1* group than low *HSP90AA1* expression (**a**) in the TCGA cases, significant differences were noted in patient survival in the *HSP90AA1* high and low groups (**b**). BC, breast cancer; BCOS, breast cancer overall survival; BCSS, breast cancer-specific survival; TCGA, The Cancer Genome Atlas.

Figure. 2

a)



b)



c)

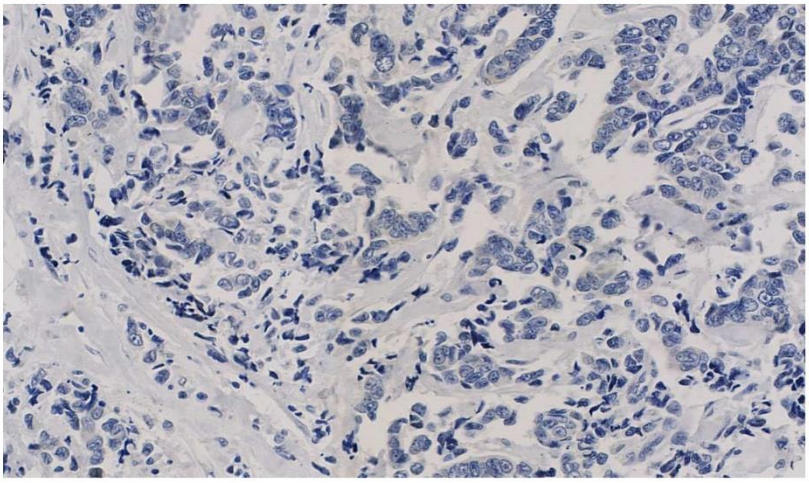
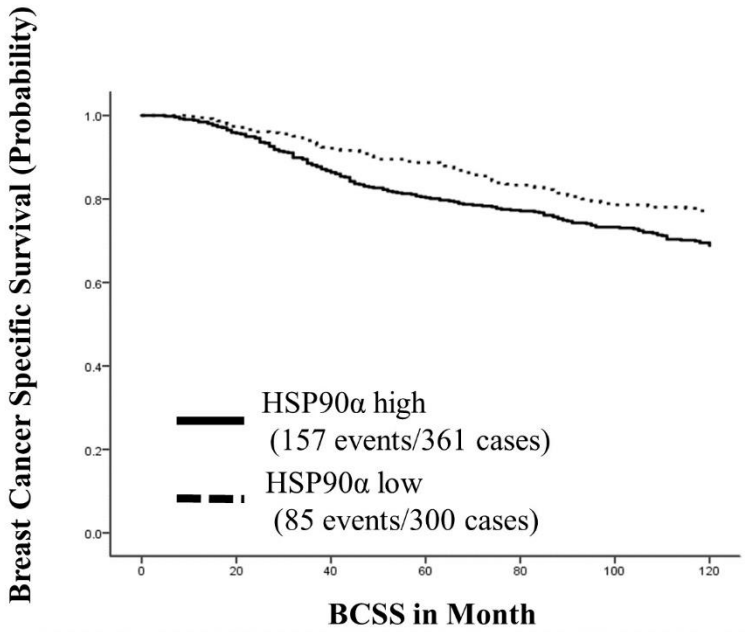


Fig. 2: HSP90α immunohistochemical protein expression in tissue microarray cores of normal and breast cancer tissue. Invasive cancer cells (strong staining in the cytoplasm of cancer cells) (**a**) normal mammary gland (uniformly weak staining) **(b**) no staining, (images are at 20x magnification) (**c**).

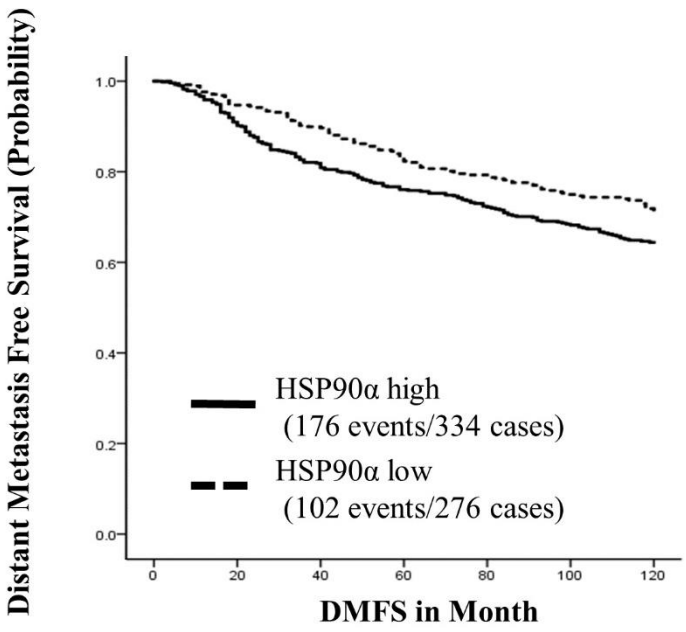
Figure. 3

a)



Hazard Ratio: 1.45; 95% CI: 1.117-1.89, p=0.005

b)



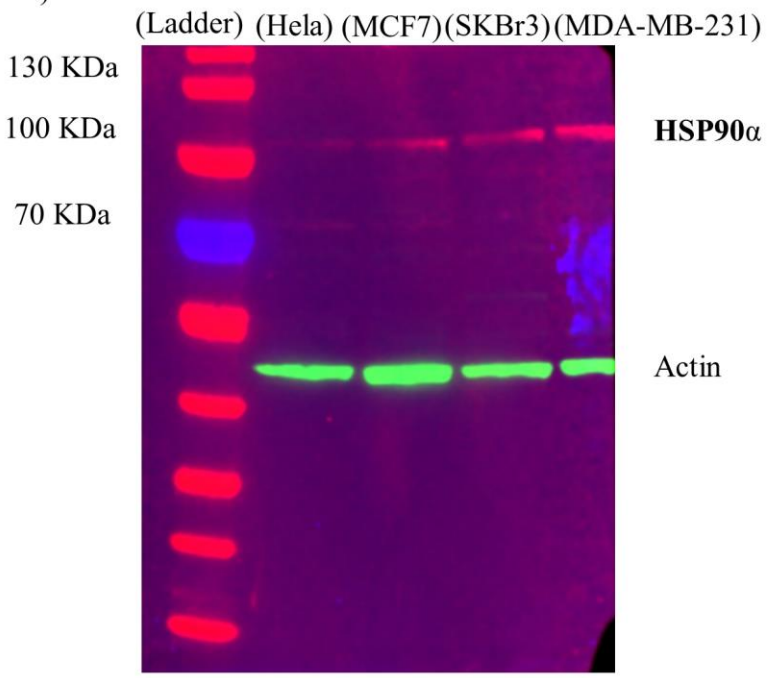
Hazard Ratio: 1.359; 95% CI: 1.65-1.735, p=0.013

Fig. 3: Cumulative survival of BC patients stratified by HSP90α protein expression.

BCSS (**a**) and DMFS (**b**) plots of cytoplasmic HSP90α revealed that high expression was associated with poor patient outcome. BC, breast cancer; BCSS, breast cancer-specific survival; DMFS, distant metastasis-free survival.

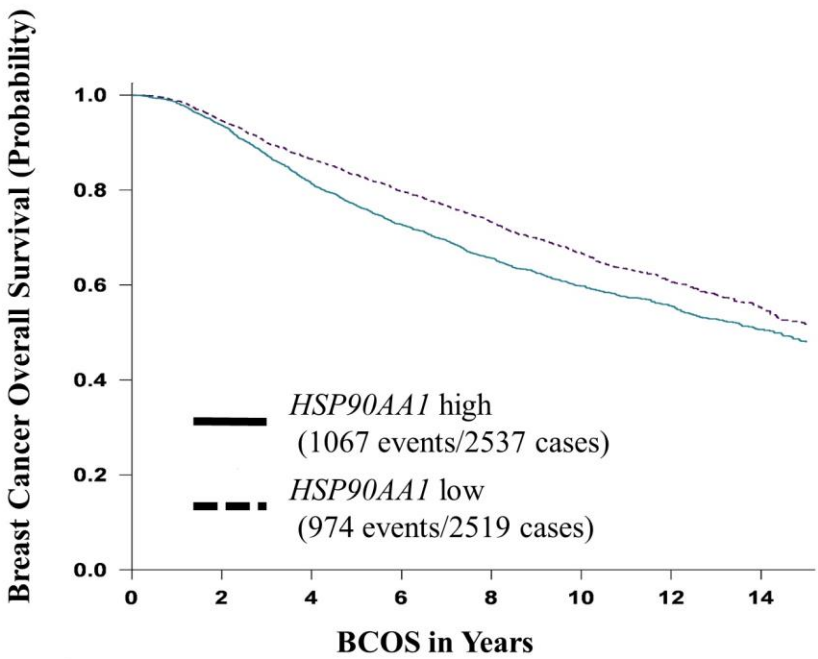
Supplementary Figure. 1

a)



Western Blot validation showed HSP90α primary antibody specificity with a single band observed at approximately 90 kDa

b)

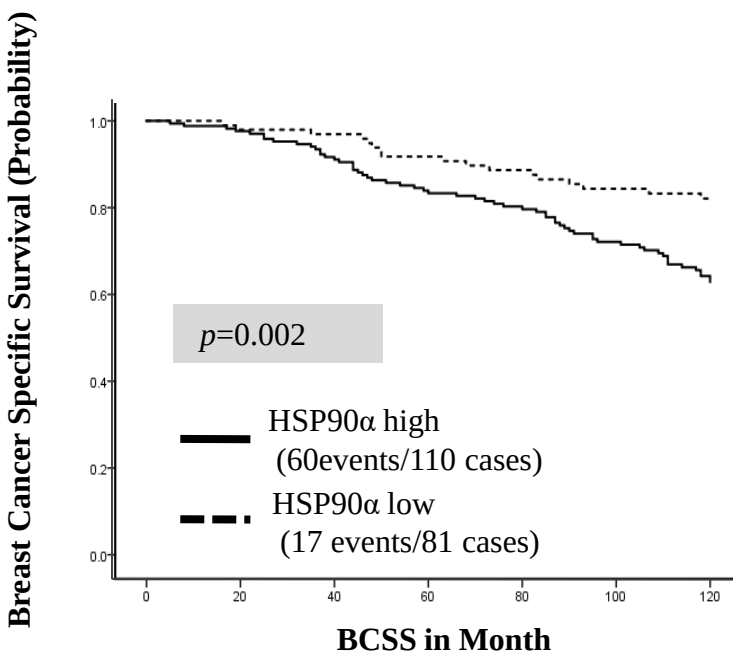


Hazard Ratio: 1.17; 95% CI: 1.07-1.28, p=0.0004

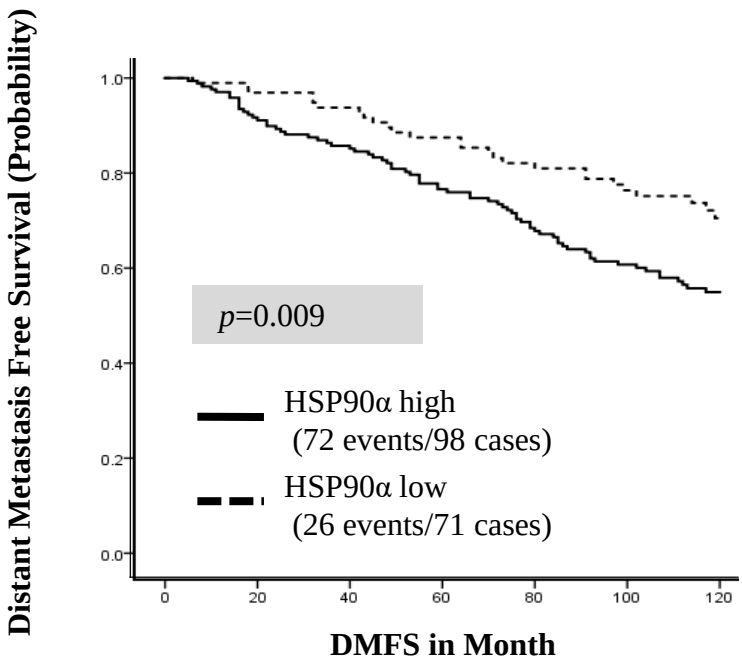
Kaplan-Meier survival plots of *HSP90AA1* mRNA expression in the BC gene miner datasets revealed that high expression was associated with poor patient outcome

Supplementary Figure. 2

a)



b)



Cumulative survival of BC patients stratified by HSP90α protein expression. Breast Cancer Specific Survival **a**) and distant metastasis free survival **b**) plots of cytoplasmic HSP90α revealed that high expression was associated with poor patient outcome in Luminal B HER2/negative subtype.

Supplementary Table 1. Clinicopathological characteristics of the Nottingham primary invasive breast cancer cohort

Age at diagnosis (years)	**Number (%)**
≤50	316 (35%)
>50	595 (65%)
Menopausal status	
Premenopausal	354 (~39%)
Postmenopausal	551 (~60%)
Tumour size	
≤2.0 cm	433 (~48%)
>2.0 cm	472 (~52%)
Tumour grade	
1	145 (~16%)
2	317 (~35%)
3	440 (~48%)
Nodal status	
Negative	559 (~61%)
Positive	347 (~38%)
Nodal stage	
1	557 (~61%)
2	276 (~30%)
3	70 (~8%)
Lymphovascular invasion	
Negative	596 (~65%)
Positive	308 (~34%)
NPI	
Good prognostic group (GPG)	277 (~30%)
Moderate prognostic group (MPG)	472 (~52%)
Pore prognostic group (PPG)	148 (~16%)
Chemotherapy	
Yes	165 (~18%)
No	709 (~78%)
Endocrine therapy	
Yes	330 (~36%)
No	546 (~60%)

Supplementary Table 2. Multivariate Cox regression analysis for predictors of Breast cancer Specific Survival (BCSS) and Distant Metastasis free Survival (DMFS) based on clinicopathological factors and protein expression of HSP90α

Factors	Multivariate analysis BCSS			Multivariate analysis DMFS		
	Hazard Ratio	**95% CI**	***p*-value**	**Hazard Ratio**	**95% CI**	***p*-value**
HSP90α expression	1.378	1.056-1.798	**0.018**	1.321	1.032-1.690	**0.027**
Tumour size	1.62	1.225-2.136	**0.001**	1.725	1.338-2.224	**<0.0001**
Axillary nodal stage	1.914	1.579-2.320	**<0.0001**	2.723	1.919-3.864	**<0.0001**
Lymphovascular invasion	1.46	1.106-1.905	**0.007**	0.647	0.387-1.081	0.096