

Gasdermin B expression predicts poor clinical outcome in HER2-positive breast cancer

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Keywords: HER2-positive breast cancer, gasdermin B, clinical behaviour, predictive biomarker, resistance to therapy

Received: December 10, 2015

Accepted: July 06, 2016

Published: July 22, 2016

ABSTRACT

Around, 30–40% of HER2-positive breast cancers do not show substantial clinical benefit from the targeted therapy and, thus, the mechanisms underlying resistance remain partially unknown. Interestingly, *ERBB2* is frequently co-amplified and co-expressed with neighbour genes that may play a relevant role in this cancer subtype. Here, using an *in silico* analysis of data from 2,096 breast tumours, we reveal a significant correlation between Gasdermin B (*GSDMB*) gene (located 175 kilo bases distal from *ERBB2*) expression and the pathological and clinical parameters of poor prognosis in HER2-positive breast cancer. Next, the analysis of three independent cohorts (totalizing 286 tumours) showed that approximately 65% of the HER2-positive cases have *GSDMB* gene amplification and protein over-expression. Moreover, *GSDMB* expression was also linked to poor therapeutic responses in terms of lower relapse free survival and pathologic complete response as well as positive lymph node status and the development of distant metastasis under neoadjuvant and adjuvant treatment settings, respectively. Importantly, *GSDMB* expression promotes survival to trastuzumab in different HER2-positive breast carcinoma cells, and is associated with trastuzumab resistance phenotype *in vivo* in Patient Derived Xenografts. In summary, our data identifies the *ERBB2* co-amplified and co-expressed gene *GSDMB* as a critical determinant of poor prognosis and therapeutic response in HER2-positive breast cancer.

INTRODUCTION

The identification of molecular cancer drivers has considerably improved clinical outcomes in oncology. As such, inhibition of the human epidermal growth factor receptor 2 (HER2; *ERBB2* gene) represents a paradigm in breast cancer oncology. Over-expression of *ERBB2* occurs in approximately 20% of breast tumours [1, 2]. Thus, the amplification of chromosome 17q12-q21, which includes *ERBB2*, defines an intrinsic molecular cancer subtype associated with relatively aggressive tumour behaviour and poor clinical outcome [3]. However, the development of targeted anti-HER2 therapies based principally on humanized antibodies (such as trastuzumab, trastuzumab emtansine -T-DM1 or pertuzumab) and tyrosine kinase inhibitors (such as lapatinib) has dramatically improved the outcome of women with early and advanced stage HER2-positive breast cancer [4–11]. Several clinical trials have demonstrated that combination with chemotherapy in the neoadjuvant or adjuvant setting significantly improves clinical response without relevant toxicity [5, 13–16]. Despite the clinical benefit obtained from the above therapies, inhibition of HER2 alone is generally not sufficient. In fact, trastuzumab response rate is limited to approximately 15–26% [12, 13], which indicates the involvement of other molecules and/or signalling pathways that influence outcome in this setting [14].

Since the common 17q12-q21 amplicon includes several gene loci, *ERBB2*-co-amplified genes have been examined for their potential to influence cancer progression and/or therapeutic response [17,18]. A minimal amplified region around *ERBB2* oncogene has been defined in several studies [19, 20], and some of the genes have been found to play a role in tumorigenesis [14, 21, 22]. These genes include *STARD3* and *GRB7*, whose products promote the proliferation of HER2-positive breast cancer cell lines [18]. Thus, *GRB7* over-expression has been identified as an independent prognostic factor [23], and the co-amplification of *ERBB2* with the non-core gene topoisomerase II (*TOP2A*) predicts sensitivity to anthracycline therapy [21, 24, 25]. Together, these observations illustrate the importance of additional genes in the biology and clinical evolution of HER2-positive breast cancer.

Previous data demonstrated that *GSDMB* lies within the evolutionary recombination hotspot closely linked to the *ERBB2* amplicon [20, 26]. The gene product belongs to the family of gasdermins, which includes three other human members [27–31]. Although these other members have been implicated in the development and progression of some diseases [32, 33], the role of *GSDMB* in cancer is only now beginning to emerge [30, 31, 34]. We recently demonstrated that *GSDMB* over-expression promotes cell motility, invasion and metastasis of breast cancer cell lines, and, intriguingly, it was found over-expressed in breast tumour samples [34]; however, the potential link with breast cancer subtypes remained unexplored.

Here, through integrated gene expression and molecular analyses, we show robust associations between *GSDMB* amplification/expression and HER2-positive status. Most importantly, further analyses show that *GSDMB* gene and protein expression predicts poor clinical outcome in HER2-positive breast cancer treated, both in the neoadjuvant and adjuvant settings. Importantly, we corroborated that *GSDMB* expression is associated with trastuzumab resistance phenotype in HER2-positive breast carcinoma cells and in Patient Derived Xenografts. *GSDMB* increases cell growth and reduces apoptosis after trastuzumab treatment in breast cancer cells. Together, our data reveals *GSDMB* as a key prognostic and predictive biomarker in HER2-positive breast cancer, highlighting new opportunities for valuable combined therapies.

RESULTS

GSDMB over-expression is associated with poor prognosis of HER2-positive breast cancer

We have previously reported that relative high expression of *GSDMB*, but no other *GSDM* genes, in breast tumours is associated with poor survival in unselected breast cancer cases [34]. To assess further the molecular and clinical significance of this observation, two independent microarray expression datasets including a total of 2,096 cases were analysed [35, 36]. Overall, high *GSDMB* expression was correlated with HER2-positive status; according to the PAM50 classifier [37] or with the reported immunohistochemical results (Supplementary Table 1).

Next, the association between *GSDMB* over-expression and 17q12-q21 amplification was evaluated. Using copy number estimates from The Cancer Genome Atlas (TCGA) dataset [36], the *GSDMB* locus was shown to be amplified in 58 out of 526 (11%) tumours. Importantly, this alteration was observed only in tumours that also presented *ERBB2* amplification (58/67; 86%); thus, none of the 459 HER2-negative cases was identified as harbouring *GSDMB* amplification. Consistent with this observation, high *GSDMB* expression levels were associated with both *ERBB2* and *GSDMB* gene amplification (Supplementary Table 1).

Given the above correlations, and the role of *GSDMB* in promoting an aggressive breast cancer cell phenotype [34], we next assessed if the over-expression of *GSDMB* could also influence the prognosis of HER2-high tumours. As shown in Figure 1, *GSDMB* over-expression in these tumours was significantly associated with poor outcome: shorter disease free survival (DSF) and distant metastasis-free survival (DMFS) in the Ur-Rehman dataset ($p \leq 0.001$, Figure 1A–1B); as well as overall survival (OS) in the TCGA dataset ($p < 0.01$, Figure 1C). Additionally, the prognosis value of *GSDMB* in these datasets was not independent of tumor grade. The association with poor prognosis was significantly stronger

(higher Hazard Ratios) in HER2-high cancers than in unselected breast cancers (whole dataset, Figure 1D).

Furthermore, we analysed the prognostic value of other 22 genes located near *ERBB2*. As shown in Figure 1D, over-expression of *GSDMB* represented the largest effect relative to poor prognosis (Figure 1D).

Complementarily, to confirm further the association of *GSDMB* expression with poor prognosis we evaluated an additional series of 58 HER2-positive (by FISH and IHC) breast cancers [38]. Again, higher *GSDMB* expression associates significantly with shorter OS (log-rank $p = 0.002$) (Supplementary Figure 1).

Collectively, consistent results across different datasets demonstrate that high *GSDMB* expression identifies the most aggressive HER2+ breast tumours.

GSDMB is amplified and over-expressed in HER2-positive breast tumours

Our *in silico* analyses indicates that *GSDMB* over-expression is associated with the HER2-positive phenotype in breast cancer. To confirm this observation, the copy number status of the *GSDMB* locus was determined by FISH (Figure 2A) in a cohort of 53 breast cancer patients including HER2-positive ($n = 29$) and

HER2-negative ($n = 24$) tumours classified according to the *ERBB2* amplification status (“discovery series” in Supplementary Table 2). Thus, *GSDMB* amplification was detected in 21 tumours, and 15 of them (71.4%) were HER2-positive cases, therefore confirming the association between the both amplifications ($p = 0.034$, Table 1, Figure 2A, panel d). In the six cases classified as HER2-negative tumours showing *GSDMB* amplification, the HER2/centromere ratio was $1.9 + 0.24$, suggesting the presence of HER2 aneuploidy [39].

Next, to determine if *GSDMB* amplification leads to over-expression of the corresponding product, an anti-*GSDMB* monoclonal antibody was developed. The antibody was able to recognize all the isoforms of *GSDMB* described [34] by Western blot analysis (Supplementary Figure 2 and Materials and Methods) and by immunohistochemistry (IHC). Thus, IHC assays revealed positive expression of *GSDMB* in 23 of the 52 cases (44.2%; Figure 2B, Table 1). Importantly, this positivity was significantly correlated with *ERBB2* gene amplification (16/23, 69.6%, $p = 0.040$, Table 1) and *GSDMB* amplification ($p < 0.001$; Table 1). In fact, intense *GSDMB* staining (Figure 2B, panel f) was found in HER2-positive tumours with clear *GSDMB* gene amplification (Figure 2A, panel d, more than 10 signals/nucleus); while

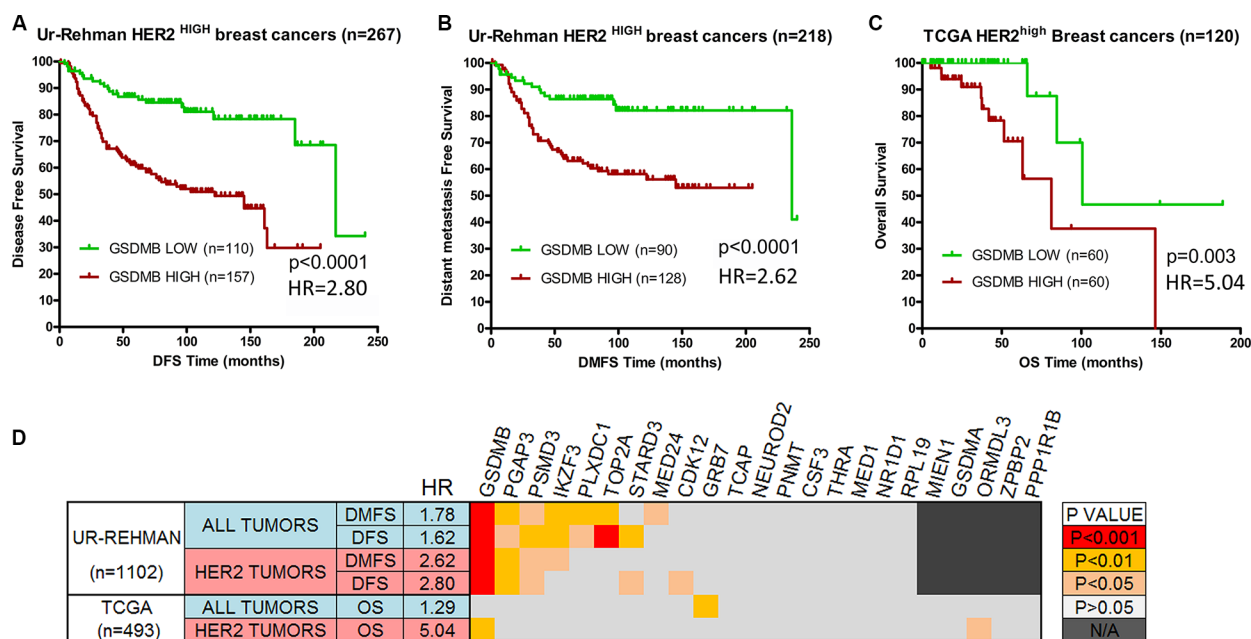


Figure 1: GSDMB over-expression is associated with poor prognosis in ERBB2-high breast cancers. Tumor samples with the top 25% expression levels of *GSDMB* gene (“high”) show significantly worse prognosis than the remaining tumors (“low”). (A) Disease-free survival and (B) Distant metastasis-free survival curves in breast cancers over-expressing (top 25%) *ERBB2* from the Ur-Rehman dataset [35]. (C) Overall patient survival curves for *ERBB2*-high tumors in the TCGA dataset [36]. Kaplan-Meier survival curves were generated and differences in survival were assessed by log-rank test ($p < 0.05$ considered statistically significant) using GraphPad PRISM 6.0. (D) Survival analyses of 23 genes close to *ERBB2* locus in breast cancers. Tumour samples with the top 25% expression levels of each gene (considered “high”) were compared to the remaining tumors (“low”). Kaplan-Meier survival curves were generated and differences in DFS and DMFS survival were assessed by log-rank Mantel-Cox test using GraphPad PRISM 6.0. Analyses were performed in the Ur-Rehman and the TCGA datasets with all tumours ($n = 1,102$ and $n = 493$, respectively), and for HER2-high (HER2+) cancers only. The p value of each test was color-coded as indicated in the scale on the right. HR: Hazard ratio associated with *GSDMB* gene high expression in each dataset.

Table 1: Relationship between GSDMB gene amplification/protein expression and HER2 oncogene in breast cancer “discovery series”

	HER2 amplification: *n (%)	
	Negative	Positive
GSDMB amplification (n = 52)		
Negative (n = 31)	18 (58.1)	13 (41.9)
Positive (n = 21)	6 (28.6)	15 (71.4)
		<i>p</i> = 0.034
GSDMB expression (n = 52)		
Negative (n = 29)	17 (58.6)	12 (41.4)
Positive (n = 23)	7 (30.4)	16 (69.6)
		<i>p</i> = 0.040
	GSDMB amplification: *n (%)	
	Negative	Positive
GSDMB expression (n = 52)		
Negative (n = 29)	29 (100)	0
Positive (n = 23)	2 (8.7)	21 (91.3)
		<i>p</i> < 0.001

*n (%), number of analyzed cases and (percentage).

undetectable or weak GSDMB staining was detected in normal breast tissue (Figure 2B, panel b) or lesions without *ERBB2* or *GSDMB* amplification (*GSDMB* gene copy number of < 4 signals/nucleus, Figure 2A, panel c).

Together, these data demonstrate that GSDMB is over-expressed in a subset (~65%) of HER2-positive tumors, with gene amplification being most likely the mechanism responsible of this alteration.

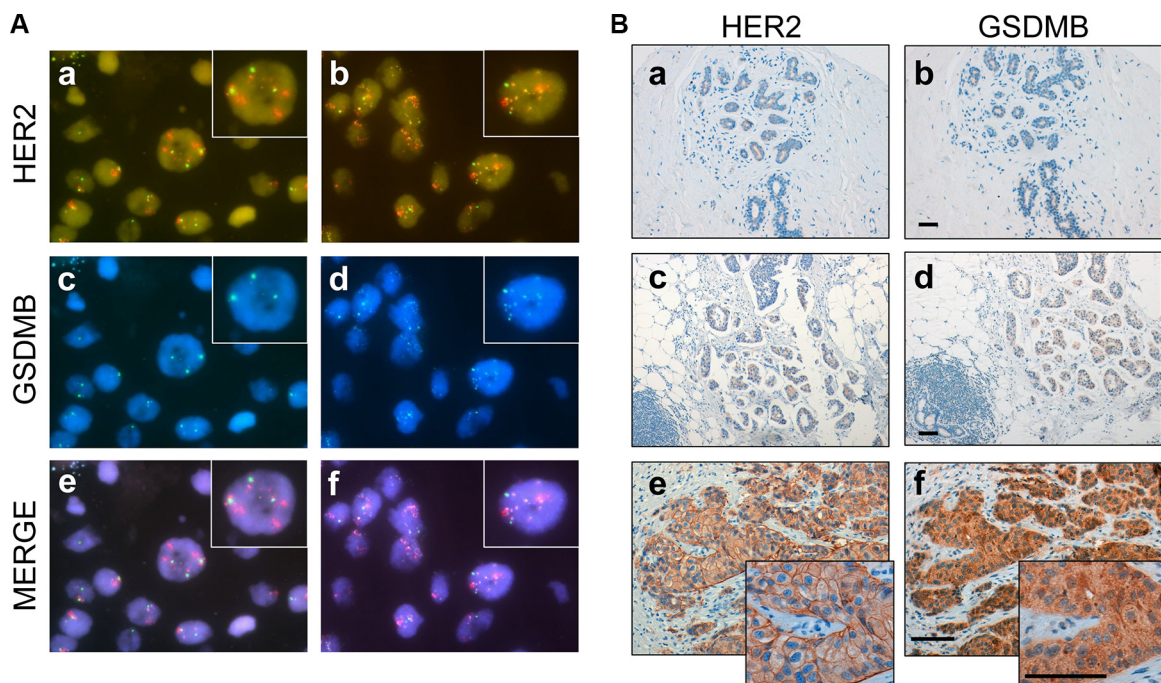


Figure 2: GSDMB gene amplification and protein expression in HER2-positive breast cancer. (A) Representative breast tumours with positive and negative *HER2* and *GSDMB* amplification. Panels a, c, e: represent an example of breast carcinoma *HER2*-positive (red signal) without *GSDMB* amplification. Panels b, d, f: represent an example of case *HER2*-positive with *GSDMB* amplification (blue signal). The lower lane shows the merged image (e of a-c and f of b-d panels). Green signals represent CEP7. Magnification x 63x; insets x 120. (B) Immunohistochemical analysis of *HER2* and *GSDMB* in normal tissue (panel a, b), and in breast carcinoma cases (panels c–f). Low or undetectable *GSDMB* staining was observed in normal breast tissue (b) or in lesions with *GSDMB* gene copy number of < 4 signals/nucleus (d) (considered non-amplified tumours). Intense *GSDMB* staining in a case with > 10 *GSDMB* copy number (f) considered as *GSDMB*-positive tumour. Scale bar measures 100 micrometers.

GSDMB gene amplification or expression predict poor clinical response and relapse under neoadjuvant settings in HER2-positive breast tumors

The results of the gene expression analyses indicate that *GSDMB* over-expression associates with poor prognosis of HER2-positive breast cancer. Next, we assessed the influence of *GSDMB* gene amplification and/or expression in the therapeutic response of this cancer subtype. First, the analysis of a series of 28 HER2-positive tumours in the discovery series, treated with the neoadjuvant setting revealed an association between *GSDMB* amplification and the lack of pathologic complete response (pCR) ($p = 0.001$, Table 2a). All of the analysed samples who developed local or distant relapse during the clinical follow-up showed *GSDMB* gene amplification and/or protein expression, respectively ($p = 0.030$, Table 2a, 2b).

In order to validate the above results, an independent cohort of 95 HER2-positive breast cancer cases treated with the same neoadjuvant regimens (“validation series”, Supplementary Table 2) was analysed. As expected, 61.1% and 68.8% of the HER2-positive tumours showed *GSDMB* amplification and protein expression, respectively (Supplementary Table 3); in fact, a strong association between both variables was observed ($p < 0.001$; Table 2a). Importantly, the analysis of this series corroborated the association between *GSDMB* gene amplification/expression and poorer therapy response. Among the 37 non-responder tumours, 31 (83.8%) showed *GSDMB* gene amplification and 33 (89.2%) protein expression (Table 2a–2b). Conversely, few cases without *GSDMB* amplification (16.2%) or protein expression (10.8%) were classified as non-responders ($p < 0.001$, Table 2a–2b). In both series, we did not find a clear association between *GSDMB* and hormone receptors expression suggesting that *GSDMB* could be a hormone receptor-independent biomarker.

Next, univariate Cox proportional hazard analyses in the validation series revealed that both variables, *GSDMB* amplification and *GSDMB* expression, are significantly associated with reduced DFS in the neoadjuvant setting: HR = 5.60, 95% CI 1.66–18.88, long-rank $p = 0.0002$; and HR = 6.75, 95% CI 1.57–28.73, long rank $p = 0.0004$, respectively (Figure 3, Table 3). The average time to relapse for the *GSDMB* amplified and non-amplified cases was 37 ± 11 and 67.3 ± 17 months, respectively (Table 3, Figure 3A). Similarly, *GSDMB* positive and negative cases identified by IHC presented an average time to relapse of 34.5 ± 14 and 66.2 ± 18 months (Figure 3B). The Kaplan-Meier and log-rank survival analyses further illustrate the significant difference in DFS between *GSDMB*-positive and *GSDMB*-negative groups (Figure 3A, 3B). Together, these results indicate that *GSDMB* expression/amplification, influences both tumour progression and the response to therapy in HER2-positive breast cancer within the neoadjuvant setting.

Clinical association of GSDMB with low therapy response in the adjuvant setting in HER2-positive breast tumours

To evaluate further the influence of *GSDMB* expression on the clinical response, a series of 138 breast cases treated with adjuvant therapy were analysed, 53 (41.1%) of these tumours were classified as HER2-positive and were treated following standard adjuvant schedules (Supplementary Table 4). In this series, around 51% of these HER2-positive samples also expressed *GSDMB* (Table 4). Next, significant associations between *GSDMB* expression and positive lymph node status ($p = 0.013$) as well as with the existence of distant metastasis ($p = 0.001$) were revealed (Table 4). Univariate Cox proportional hazard analysis confirmed the association with DFS: HR = 2.85, 95% CI 1.28–6.37, log-rank $p = 0.007$ (Table 5). Thus, the average time to relapse for the *GSDMB*-positive and -negative cases was $91.24 + 14.88$ and $101.81 + 9.51$ months, respectively. The Kaplan-Meier and log-rank survival analyses further illustrate the difference in DFS between *GSDMB*-positive and *GSDMB*-negative cases in the adjuvant setting ($p = 0.007$; Figure 3C).

Overall these results and our previous data firmly establishes *GSDMB* as a negative prognostic marker in HER2-positive breast cancer patients treated (trastuzumab plus chemotherapy) in both the neoadjuvant and adjuvant settings.

GSDMB promotes survival to trastuzumab treatment

Finally, to assess if *GSDMB* expression was functionally involved in promoting survival to anti-HER2 therapy we performed several complementary experimental approaches. First, we evaluated the potential role of *GSDMB* expression on trastuzumab response in HCC1954 cells in which endogenous *GSDMB* has been stably silenced by shRNAs [34]. This model is an example of trastuzumab-resistant HER2+ human breast cancer cell line [40]. Additionally, we generated *GSDMB* exogenously expressing cells using SKBR3 (Figure 4A), which are trastuzumab-responsive cells [40]. In both cell models, trastuzumab inhibition of cell growth (measured by Alamar Blue method) was significantly higher (~20%) in cells with low *GSDMB* expression (Figure 4B–4C). Moreover, *GSDMB* significantly increased cell survival to trastuzumab treatment, as demonstrated by the reduction in apoptosis in *GSDMB*-overexpressing SKBR3 cells compared to control cells (Figure 4D). Given that HCC1954 are intrinsically very resistant to trastuzumab (5 mg/ml) we could not test the effect on apoptosis in this cell line.

Second, to demonstrate the association of *GSDMB* with resistance *in vivo* we utilized six breast cancer Patient Derived Xenografts (PDX), a more clinically relevant model [41]. We used four HER2-positive breast cancer PDXs, two sensitive and two primary resistant to trastuzumab

Table 2: Relationship between GSDMB amplification/expression and clinico-pathological features in HER2-positive breast cancer included in the discovery ($n = 28$) and validation series ($n = 95$)

(a)	Discovery series		Validation series	
	GSDMB amplification: *n (%)		GSDMB amplification: *n (%)	
	Negative	Positive	Negative	Positive
ER expression				
Negative	6/9 (66.7)	3/9 (33.3)	11/35 (31.4)	24/35 (68.6)
Positive	7/19 (36.8)	12/19 (63.2)	26/60 (43.3)	34/60 (56.7)
		$p = 0.142$		$p = 0.176$
PR expression				
Negative	10/19 (52.6)	9/19 (47.4)	16/49 (32.7)	33/49 (67.3)
Positive	3/9 (33.3)	6/9 (66.6)	19/44 (43.2)	25/44 (56.8)
		$p = 0.338$		$p = 0.295$
GSDMB expression				
Negative	12/12 (100)	0 (0)	29/29 (100)	0 (0)
Positive	1/16 (6.2)	15/16 (93.8)	6/64 (9.4)	58/64 (90.6)
		$p < 0.001$		$p < 0.001$
pCR †				
Responders	12/16 (75.0)	4/16 (25.0)	31/58 (53.4)	27/58 (46.6)
Non-responders	1/12 (8.3)	11/12 (91.7)	6/37 (16.2)	31/37 (83.8)
		$p = 0.001$		$p < 0.001$
Relapse †				
Negative	12/26 (57.1)	9/26 (42.9)	24/47 (51.1)	23/47 (48.9)
Positive	0 (0)	5/5 (100)	3/19 (15.8)	16/19 (84.2)
		$p = 0.030$		$p = 0.008$

(b)	GSDMB expression: *n (%)		GSDMB expression: *n (%)	
	Negative	Positive	Negative	Positive
	ER expression			
Negative	6/9 (66.7)	3/9 (79.5)	9/35 (25.7)	26/35 (74.3)
Positive	6/19 (31.6)	13/19 (68.4)	20/58 (34.5)	38/58 (65.5)
		$p = 0.090$		$p = 0.145$
PR expression				
Negative	9 (47.4)	10 (52.6)	13/48 (27.1)	35/48 (72.9)
Positive	3 (33.3)	6 (66.7)	14/43 (32.6)	29/43 (67.4)
		$p = 0.293$		$p = 0.366$
pCR†				
Responders	11 (68.8)	5 (31.3)	25/56 (44.6)	31/56 (55.4)
Non-responders	1 (8.3)	11 (91.7)	4/37 (10.8)	33/37 (89.2)
		$p = 0.002$		$p < 0.001$
Relapse†				
Negative	11 (52.4)	10 (47.6)	21/46 (45.7)	25/46 (54.3)
Positive	0	5 (100)	2/19 (10.5)	17/19 (89.5)
		$p = 0.033$		$p = 0.006$

The data refers to the available cases for each markers (a) Statistical analysis of GSDMB amplification in breast carcinoma samples included in discovery and validation series. (b) Statistical analysis of GSDMB IHC study in breast carcinoma samples included in discovery and validation series. *n (%), number of analyzed cases and (percentage). †pCR: pathological complete response when there is no invasive presence of tumour at the breast or ganglia level (< 0.1 mm) and Relapse as local or distant recurrence in HER2-positive tumours.

Table 3: Risk of relapse in HER2-positive tumour validation series according to GSDMB copy number or GSDMB immunohistochemistry results

Evaluation	Non-relapsed *n, %	Relapsed *n, %	Average time to relapse (months)	HR	95% CI	p
FISH GSDMB (n, %)						
Amplified (37, 56.1%)	16 (43.2%)	21 (56.8%)	37.0 ± 11	5.60	1.66–18.88	0.0002
Non-amplified (29, 43.9%)	26 (89.7%)	3 (10.3%)	67.3 ± 17			
IHC GSDMB (n, %)						
Positive (42, 63.6%)	20 (47.6%)	22 (52.4%)	34.5 ± 14	6.75	1.57 – 28.77	0.0004
Negative (23, 34.8%)	21 (91.3%)	2 (8.7%)	66.2 ± 18			

Table 4: Relationship between GSDMB over-expression and clinic pathological features in adjuvant breast cancer series

	GSDMB expression †n (%)	
	Negative	Positive
HER2 amplification (n = 127)		
Negative (n = 74)	68 (91.9)	6 (8.1)
Positive (n = 53)	26 (49.1)	27 (50.9)
		p < 0.001
ER expression (n = 43) *		
Negative (n = 15)	12 (80.0)	3 (20.0)
Positive (n = 28)	11 (39.3)	17 (60.7)
		p = 0.012
PR expression (n = 49) *		
Negative (n = 13)	8 (61.5)	5 (38.5)
Positive (n = 36)	17 (47.2)	19 (58.2)
		p = 0.489
Lymph node status (n = 49)*		
Negative (n = 22)	16 (72.7)	6 (27.3)
Positive (n = 27)	10 (37.0)	17 (63.0)
		p = 0.013
Distant metastasis (n = 46) *		
Negative (n = 30)	22 (73.3)	8 (26.7)
Positive (n = 16)	3 (18.8)	13 (81.2)
		p = 0.001

*Only evaluated in HER2-positive breast carcinomas. †n (%), number of analyzed cases and (percentage).

([42] and data not shown) and two HER2-negative PDX as control (Figure 4E). The PDXs that did not respond to trastuzumab *in vivo* exhibited higher mRNA (Figure 4E) and protein levels (Supplementary Figure 3) of GSDMB than trastuzumab-sensitive patients and HER2-negative PDXs. Next, one of the trastuzumab-sensitive PDX (PDX118) was treated *in vivo* (biweekly; 1 mg/ml) with trastuzumab to induce an acquired drug resistance [43]. In most of the resistant PDXs originated from PDX118, we observed a significant increase in GSDMB (but not HER2) levels, thus corroborating that its expression is specifically associated with *in vivo* resistance to trastuzumab (Figure 4F).

Altogether, these data demonstrate that GSDMB overexpression is functionally involved in promoting resistance to the anti-HER2 immunotherapy trastuzumab, and this may explain its association with adverse clinical behaviour in HER2+ cancers.

DISCUSSION

Despite significant clinical benefits to trastuzumab in HER2-positive breast tumors, not all patients respond well to this therapy, and therefore experience disease recurrence and progression [44, 45], within a few months

of therapy (median of 7.4 months) [8,13]. In this scenario, the mechanisms of resistance to HER2-targeted therapy are not completely understood [46], and this limitation includes the unknown role of 17q12-q21 genes linked to *ERBB2*.

Our study demonstrates that gasdermin B (*GSDMB*), mapped 175 kilo bases distal to *ERBB2*, is amplified and

over-expressed in a subset (~60%) of HER2-positive breast tumours. Importantly, *GSDMB* gene and protein over-expression is significantly associated with poor clinical outcome, in terms of disease progression, relapse and response after neoadjuvant therapy (Table 2), as well as lymph node positivity and the development of distant

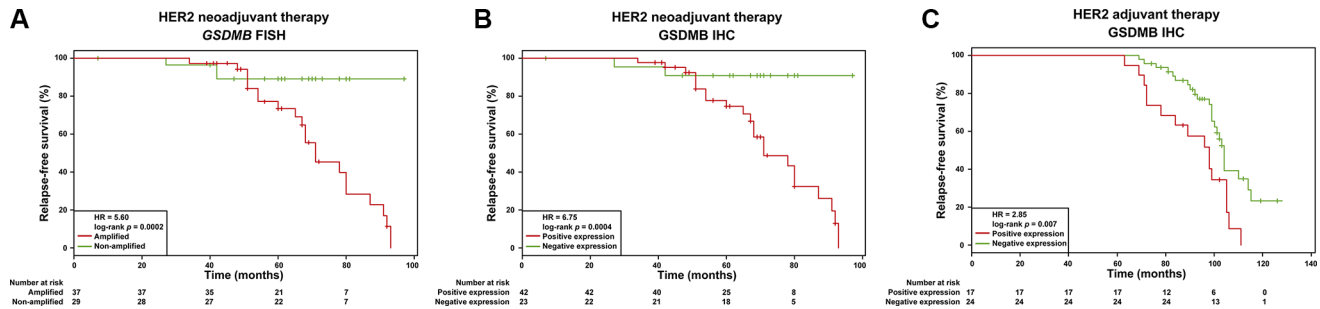


Figure 3: GSDMB gene amplification and/or protein expression associates with relapse in HER2-positive breast cancer samples under neoadjuvant and adjuvant treatment regimens. Relapse-free survival curves in HER2-positive breast cancer patients ($n = 95$) treated with neoadjuvant settings in relation to (A) *GSDMB* gene amplification ($p = 0.0002$) and (B) *GSDMB* expression scored by immunohistochemistry (IHC, $p = 0.0004$). (C) Relapse-free survival curves in HER2-positive breast cancer patients ($n = 53$) treated with adjuvant settings in relation to *GSDMB* protein expression measured by IHC ($p = 0.007$). Statistical differences, HR and p -value, were calculated via log-rank test. Red line: *GSDMB* amplified and expressed patients, green line: patients without *GSDMB* amplification and expression.

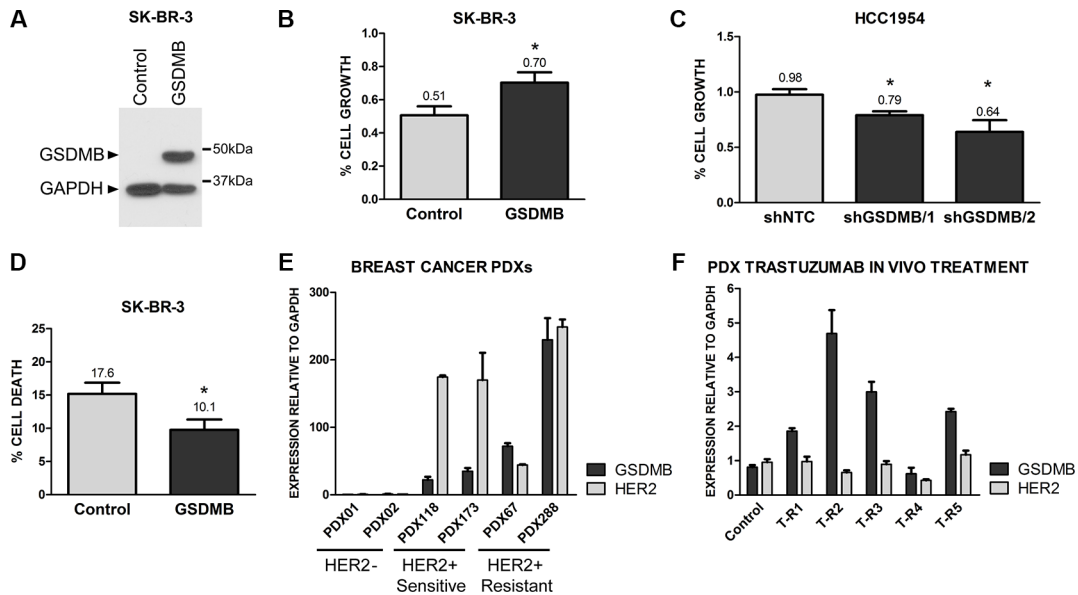


Figure 4: GSDMB expression increases survival to trastuzumab treatment. (A) Analysis of *GSDMB* expression by western blot in SKBR3 cells (empty vector, Control) and *GSDMB*-overexpressing cells (*GSDMB*). GAPDH was used as a loading control. (B) Analysis of cell growth (measured with Alamarblue) in control and *GSDMB*-overexpressing SKBR3 cells treated with 1 mg/ml trastuzumab for 72 h; (C) Reduction of cell growth in control (shNTC) and *GSDMB*-silenced (two shRNAs) HCC1954 cells treated with 5 mg/ml trastuzumab. (D) Analysis of cell death (measured with Annexin V and propidium iodide) in control and *GSDMB*-overexpressing SKBR3 cells 72 h after trastuzumab treatment. Bars represent mean \pm SEM of three independent experiments. (E) *GSDMB* and *HER2* mRNA expression in Patient Derived Xenografts (PDX) from *HER2*-negative (PDX01, 02) and *HER2*-positive breast carcinomas. PDXs were classified as trastuzumab sensitive (PDX118, 173) or resistant (PDX67, 288) according to *in vivo* patient response [42]. (F) *GSDMB* and *HER2* mRNA expression in a trastuzumab-sensitive PDX (PDX118) and its trastuzumab-resistant derived PDXs (T-R1-R5), obtained by chronic *in vivo* treatment with trastuzumab. mRNA values were normalized using GAPDH as housekeeping gene. Bars represent mean \pm SEM expression of three experiments by quantitative PCR.

Table 5: Risk of relapse in HER2-positive breast tumours treated under adjuvant regimens according to GSDMB immunohistochemistry results

Evaluation	Non-relapsed *n, %	Relapsed *n, %	Average time to relapse (months)	HR	95% CI	p
IHC GSDMB (n, %)						
Positive (28, 45.2%)	7 (11.3%)	21 (33.9%)	91.24 ± 14.88	2.85	1.28–6.37	0.007
Negative (34, 54.8%)	17 (27.4%)	17 (27.4%)	101.8 ± 9.51			

metastases in the adjuvant context (Table 4) independently of hormone receptors status. Notably, the estimated effect in the neoadjuvant setting is relatively high (HR > 5) and, hence, the average time to relapse is almost halved in cases with *GSDMB* amplification or *GSDMB* over-expression.

GSDMB belongs to the gasdermin family of proteins [47]. Four gasdermin (*GSDM*) gene members have been identified in humans (*GSDMA-D*) [48]. *GSDM* genes have been involved in secretion, proliferation and differentiation in various tissues [48], although their major role seems to be the regulation of diverse types of cell death. In fact, *GSDMD* has been reported to be a substrate of caspase 11, essential for the activation of pyroptosis as anti-bacterial innate immune defence [49–52] and *GSDMA* promotes cell death through autophagy and mitochondrial damage [49,50]. The cell-death promoting function of *GSDMA*, C and D, depends on the cleavage of their N-terminal portion [51]. Interestingly, *GSDMB* does not induce cell death, and its C-terminal region is able to reduce partially the cell death induced by *GSDMA* [50]. Consistently, our data in different breast cancer cell lines models as well as in tumor Patient Derived Xenografts (PDXs) indicate that *GSDMB* promotes cell survival to trastuzumab anti-HER2 immunotherapy. Given that *GSDMB* alone does not affect cell growth [34], these results point out that *GSDMB* might be a negative regulator of cell death/survival under certain conditions (anti-HER2 therapy). Additionally, we have previously shown that *GSDMB* over-expression in a breast cancer cell line increased tumorigenic and invasive behaviour [34]. Therefore, *GSDMB* is functionally involved in promoting aggressive tumour behaviour and reduced clinical response to anti-HER2 therapies. It would be of interest in the future to decipher the exact molecular mechanisms by which *GSDMB* mediates these effects.

In conclusion, our study has identified *GSDMB* as a relevant biomarker of poor prognosis that is also able to predict non-response to current standard therapy for HER2-positive breast cancer. Since *GSDMB* expression promotes cell survival to anti-HER2 treatment, the analysis of *GSDMB* gene status and/or *GSDMB* expression may lead to more precise molecular classification of this cancer subtype and, in turn, help to identify cases that may not benefit from the current therapies.

MATERIALS AND METHODS

Tumour samples

A total of 286 paraffin-embedded ductal breast carcinoma included in this study was collected between 2003 and 2014 at the Vall d’Hebron Hospital (Barcelona), Virgen del Rocío Hospital (Seville, Spain) and the MD Anderson Cancer Centre (Madrid). This study was approved by the local ethical committee from each institution, and a complete written informed consent was obtained from all patients. Briefly, this study was performed in two independent data samples treated under neoadjuvant treatment: discovery series, which included 29 HER2 positive and 24 HER2 negative breast carcinomas, and validation series composed by 95 HER2 positive breast carcinomas (Supplementary Tables 2 and 3). The median age of patients and tumour size was 53.2 ± 14.9 years and 2.23 ± 1.45 cm, respectively. Additionally, we studied 138 high-grade ductal breast tumours (52 HER2-positive and 86 HER2-negative samples) all of them treated with adjuvant schedules (Supplementary Table 4). The mean age of patient and tumour size at surgery was 59.6 ± 9.1 years and 1.69 ± 0.42 cm, respectively. HER2 positivity was considered when the tumours showed gene amplification. All cases were treated with trastuzumab plus chemotherapy (based on a combination of anthracyclines and taxanes). Hormone therapy was added in those cases with positive hormone receptor expression. The clinical response of HER2-positive cases in the neoadjuvant setting was evaluated using the pathological complete response criteria (pCR). The pCR was assessed at the breast parenchyma and at the ganglia level. A patient will be deemed to be in pCR when there is no invasive presence of tumour at the breast or ganglia level.

Immunohistochemistry (IHC) and Fluorescence *in situ* hybridization (FISH) analysis

HER2, ER (oestrogen receptor) and PR (progesterone receptor) were analysed by IHC in TMAs and in a complete section samples from PDXs, according to standard methods as previously published [40]. *GSDMB* monoclonal antibody was generated as

described in Supplementary Information, and GSDMB expression was classified as positive when more than 10% of tumour cells showed positive cytoplasmic staining. All studied tumour sections also included normal breast tissue as an internal control. In negative controls, the primary antibodies were omitted. FISH study was performed as previously described [53]. Briefly, the analysis was carried out simultaneously using red-labelled *ERBB2*, green-labelled chromosome enumeration probe (CEP17) as control (PathVysion Kit, Abbott Molecular), and aqua-labelled *GSDMB*. The *GSDMB* probe was generated from DNA isolated from the bacterial artificial clone (BAC) RP11-387H17 obtained from the BACPAC Resource Centre (BPRC) at the Children's Hospital Oakland Research Institute (CA, USA) and labelled by nick translation. Fluorescence signals were scored in each sample by counting the number of single copy genes and control probe signals in an average of 180 (100–210) well-defined nuclei. The ASCO CAP 2007 criteria for *ERBB2* gene amplification [39] were used to evaluate the status of *GSDMB* gene. Increased gene copy number and amplification were defined as the presence of 4–6 and > 6 signals, respectively. The *GSDMB*/CEP17 ratio used to delimit amplification was of ≥ 2.2 [39].

***GSDMB* expression analysis in breast cancer microarray datasets**

To evaluate the expression of *GSDMB* in breast tumours, several independent datasets were analyzed [35, 36, 38] Microarray and clinical data were obtained from the ICR database (www.rock.icr.ac.uk). For each gene, normalized expression was categorized as “high” when it was above the third quartile (top 25% expression) of all tumour samples; otherwise, it was categorized as “low”. Kaplan-Meier estimates of *GSDMB* expression (assessed separately for each stratum) were plotted and compared to overall survival (OS), disease-free survival (DFS) and metastasis-free survival (MFS) curves using log-rank and Chi-square tests.

Cell lines and Patient Derived Xenografts (PDXs)

For the study of the role of *GSDMB* in response to trastuzumab two well-studied HER2-positive breast cancer cell lines [40], HCC1954 and SKBR3, were used. Both cell lines were obtained from the American Type Cell Culture (ATCC). Cells were grown as monolayer cultures at 37°C in an atmosphere with 5% CO₂ and authenticated by STR-profiling according to ATCC guidelines. For the analysis of *GSDMB* over-expression, SKBR3 cells were stably transfected using Lipofectamine (Invitrogen) with pEZ-M61-GSDMB (Genecopoeia) or the empty vector. Cells containing the plasmids were selected with G418 (0,8 mg/ml) treatment. *GSDMB*-silenced HCC1954 cells

were obtained as previously reported [34]. HER2-positive and trastuzumab resistant Patient Derived Xenografts (PDXs) were previously described in [42, 43]. HER2-negative PDXs were obtained from primary triple-negative breast tumours after engrafting orthotopically in the mammary fat of female athymic (nu/nu) mice (Harlan™). The patients provided written informed consent and the study was approved by IDIBELL Ethics committee. All ortho-xenograft models were established for > 3 passes. Their triple negative status was confirmed following standard immunohistochemical assays.

Cell-based assays

Trastuzumab anti-proliferative effects were measured in 2×10^3 cells plated on 96-well-plates using alamarBlue assay (Thermo Scientific) according to the manufacturer's protocol. HCC1953 and SKBR3 cells were daily treated up to 72 h with 5 mg/ml and 1 mg/ml trastuzumab (Roche Diagnostics GmbH, Penzberg, Germany), respectively, or were left untreated. For detection of the death rate, SKBR3 cells were pre-treated in 0,1% FBS-containing medium during 24 h, and then (50×10^3 .000 cells) cells were cultured in 6-well plates and treated with or without trastuzumab (1 mg/mL dissolved in medium) for 72 hours. Cell death was measured by staining with Annexin V and propidium iodide (PI) using PE-Apoptosis Detection kit (Immunostep) according to the manufacturer's instructions, and analyzed on a FACSCANTO II flow cytometer (BD Biosciences). Three independent experiments were performed for Alamar blue and Cell death measurements.

Gene expression assays

Gene expression was performed after extraction of total RNA from PDXs using RNA miniprep system, ReliaPrep™ FFPE total (Promega) following manufacturer recommendations. cDNA was obtained from 1 µg of total RNA using random primers and M-MLV reverse transcriptase (Amresco) as manufacturer recommendations. *GSDMB*, *HER2*, and *GAPDH* gene expression levels were measured by quantitative real time RT-PCR (qRT-PCR) using pre-designed TaqMan probes (ThermoFisher) and PerfeCTa FastmixII (Quanta BioSciences, Inc), on an iQ5 iCycler Realtime PCR Detection System (BioRad), according to the manufacturer's recommendations. All qRT-PCRs were performed in triplicate. Relative *GSDMB* and *HER2* expression was normalized to *GAPDH*.

Statistical analysis

The χ^2 or Fisher's exact tests were used to test associations between categorical variables. All tests were two-tailed and 95% confidence intervals (CIs) were used.

Values of $p < 0.05$ were considered statistically significant. Hazard ratios (HRs) and 95% CIs were estimated from univariate or multivariate Cox proportional hazards models. Disease-free survival was defined as the time from the date of diagnosis to the date of recurrence or development of novel distant metastasis. Survival curves were generated using the Kaplan-Meier method. The p -values are based on the Wald test for Cox proportional hazards models, and the log-rank test for Kaplan-Meier analysis. Statistical analysis were performed in R and using the Survival package and SPSS Statistics 17.0 (SPSS Inc., Chicago, IL).

ACKNOWLEDGMENTS

We thank all the people of Immunohistochemical Laboratory from MD Anderson Madrid and B16 lab from Biochemistry Department of Universidad Autónoma de Madrid for their invaluable help. Tissue samples were obtained with the support of MD Anderson Foundation Biobank (record number B.0000745, ISCIII National Biobank Record), Biobank- Hospital Virgen del Rocio (PT13/0010/0056)

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interests.

GRANT SUPPORT

This work has been supported by the Spanish Ministry of Economy and Competitiveness, “Fondo Europeo de Desarrollo Regional (FEDER), una manera de hacer Europa”, the Spanish Ministry of Health, Instituto de Salud Carlos III (ISCIII) (grants PI13/00132 and RETICC RD12/0036/0007 to GMB; PI15/00854 and RETICC RD12/0036/0008 to MAP; RD12/0036/0057 to JC; RD12/0036/0057 to JA, RD12/0036/0064 to JP), the Community of Madrid (grant S2010/BMD-2303 to GMB), the Generalitat de Catalunya (grant SGR 2014–364) to MAP, the AVON Foundation to GMB, the Breast Cancer Research Foundation (BCRF) to JA. Alba Mota is a predoctoral student supported by a FPU fellowship (Spanish Ministry of Education, Culture and Sport). David Sarrio is a postdoctoral researcher funded by the AECC Scientific Foundation.

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