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## Loss of Plexin B1 is highly prognostic in low proliferating ER positive breast cancers – Results of a large scale microarray analysis

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### ARTICLE INFO

#### Article history:

Received 28 March 2008

Received in revised form 19 August 2008

Accepted 15 October 2008

Available online 4 December 2008

#### Keywords:

Breast cancer

Microarray

Plexin

Prognosis

### ABSTRACT

Plexins, cell-surface receptors for semaphorins, are involved in cell adhesion and migration. In the previous work, we demonstrated that the loss of Plexin B1 expression is associated with poor outcome in breast cancer patients. The goal of the present study was a validation of Plexin B1 expression in a large scale microarray dataset from  $n = 1086$  breast cancer patients. Plexin B1 correlates with ER status ( $p < 0.001$ ) and is of prognostic significance only in ER positive ( $p = 0.024$ ) but not in ER negative samples ( $p = 0.85$ ). Among ER positive tumours, the loss of Plexin B1 expression is associated with a positive ErbB2 status ( $p = 0.05$ ) and a high Ki67 expression ( $p = 0.016$ ) in univariate analysis. Multivariate Cox regression including all standard parameters among ER positive tumours revealed that Plexin B1 (HR 1.59, 95% confidence interval (CI) 1.03–2.47,  $p = 0.036$ ) remains a significant prognostic marker besides tumour size (HR 2.27, 95% CI 1.33–3.89,  $p = 0.0028$ ) and Ki67 (HR 1.78, 95% CI 1.12–2.84,  $p = 0.0149$ ). Interestingly, the prognostic value of Plexin B1 was pronounced in low proliferating ER positive tumours otherwise characterised by a low risk of recurrence. In conclusion, this study confirms our previous observations suggesting Plexin B1 as a new prognostic marker in ER positive breast cancers.

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## 1. Introduction

Plexins, cell-surface receptors for semaphorins, are widely expressed in diverse epithelial cells and are involved in cell adhesion and migration.<sup>1,2</sup> They belong to the c-Met family of scatter factor receptors but lack an intrinsic tyrosine kinase domain. There is a growing evidence that Plexin B1, the receptor for semaphorin Sema4D, is involved in invasive growth by

cell-cell dissociation, anchorage-independent growth and branching morphogenesis.<sup>3</sup> Several groups could demonstrate that semaphorins are essential for the development of non-neural tissues like heart,<sup>4–6</sup> lung,<sup>7</sup> mammary gland<sup>8</sup> and bone homeostasis.<sup>9</sup> Moreover, the autocrine semaphorin-Plexin signalling seems to have tumour suppressor function in normal epithelial cells and a loss of heterozygosity of these genes could enhance the deregulation of tumour cell

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adhesion and increase migration.<sup>10–12</sup> In contrast, the observation that Plexin B1 couples with the receptor tyrosine kinases MET<sup>3</sup> and ERBB2<sup>13</sup> might suggest that Plexin B1 may trigger the invasive growth of epithelial cells.<sup>3</sup> Recently, we have demonstrated that breast cancers with low Plexin B1 expression levels characterise a more aggressive tumour phenotype resulting in an impaired prognosis in the ER positive subgroup.<sup>14,15</sup> Since these data were obtained from a limited number of samples, we performed an independent validation in nine Affymetrix microarray datasets representing a total of  $n = 1086$  breast cancer patients. Our analysis validated in this larger cohort that the loss of Plexin B1 is associated with poor outcome.

## 2. Materials and methods

### 2.1. Breast cancer samples and published microarray datasets

We established a database consisting of 1086 Affymetrix microarray samples from primary breast cancer patients without neoadjuvant treatment. For reasons of comparability, only data from Affymetrix HG-U133A microarrays were used. We included 220 samples from our own institutions (datasets Frankfurt and Hamburg), which had been described previously (Rody et al., 2007<sup>14</sup>; Ruckhäberle et al., 2007<sup>16</sup>; Rody et al., 2006<sup>17</sup>; Ahr et al., 2002<sup>18</sup>), as well as 866 samples from seven different publicly available datasets (Table 1): Stockholm,<sup>19</sup> Oxford-untreated,<sup>20</sup> Oxford-Tamoxifen and London,<sup>21</sup> New York,<sup>22</sup> Villejuif,<sup>23</sup> and ExpO.<sup>24</sup> The clinical characteristics of the patients in the different datasets are given in Table 1. Seven-hundred and ninety-two of the 1086 samples were ER positive. Treatment information could be obtained for 505 ER positive and 143 ER negative patients. Follow-up information was available for 768 patients. The median follow-up time was 67 months. Since the methods of Affymetrix microarray normalisation can have significant effects on the levels for individual probe sets, several uniform normalisation methods<sup>25,26</sup> of CEL file data have been developed to allow the analysis of sets of multiple arrays. However, important discrepancies between different datasets depend on the dynamics of the measurements originating from different hybridisation efficiencies. Unfortunately, even uniform normalisation methods are incapable in compensating those experimental differences. Therefore, in the analysis presented here we used a conservative strategy for dataset stratification by relying on a ranking of samples in each cohort. Each dataset of microarrays was normalised separately using the originally proposed method in the respective study (see Table 1). Log transformed expression values were median centred over each array. For genes, the normalisation, ranking of expression values and median splits were done separately in each dataset.

### 2.2. Assessment of ER, ErbB2, proliferative status and Plexin B1 expression of the samples

To allow comparison of different datasets and since standard pathology for ER and ErbB2 was not available for all samples, receptor status was determined based on Affymetrix expres-

Table 1 – Clinical characteristics of breast cancer patients from Affymetrix microarray datasets used in this study.

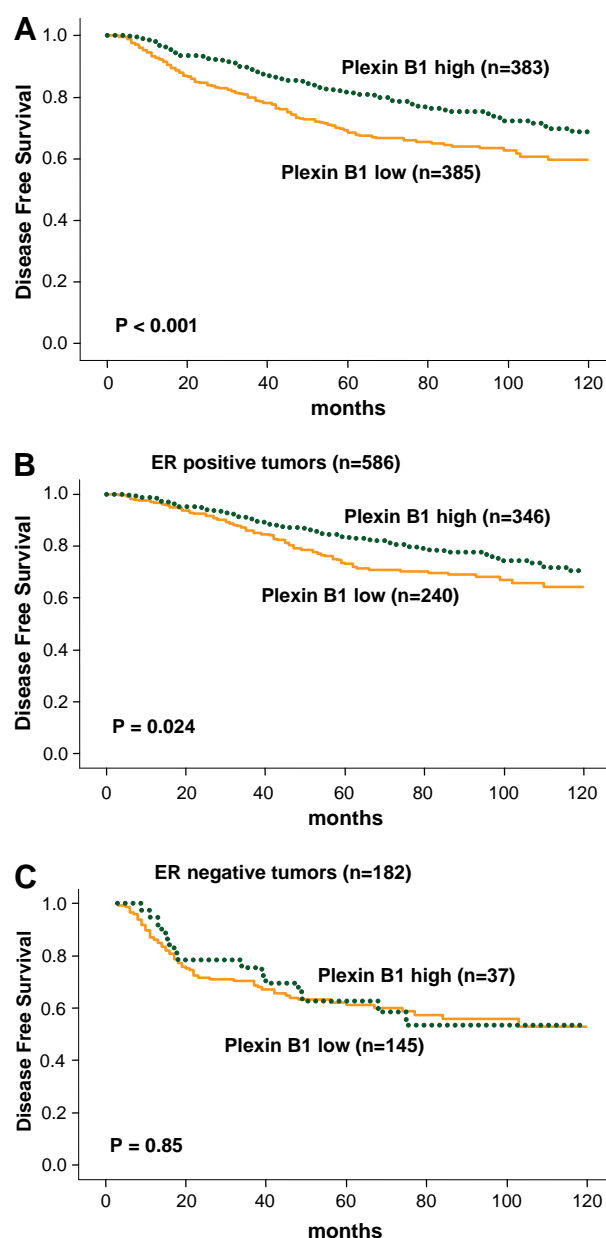
Dataset	Data source	Array	Normalisation method	Number of samples	% of samples			System treatment	Median follow up months	Number of relapses	Reference
					Age ≤ 50	Tumour size ≤ 2 cm	ER positive				
Frankfurt	This study	U133A	MAS5	120	54	50	66	Chemotherapy	39	29	[14]
Hamburg	This study	U133A	MAS5	100	46	24	65	Chemotherapy	57	31	[17]
Stockholm	GSE1456	U133A	MAS5	159	n.a.	n.a.	82	Yes/no	85	40	[19]
Oxford-untreated	GSE2990	U133A	RMA	61	44	64	69	Untreated	121	29	[20]
Oxford-Tamoxifen	GSE6532	U133A	RMA	109	14	34	95	Endocrine	61	30	[21]
London	GSE6532	U133+	RMA	87	6	35	33	Endocrine	137	28	[21]
New York	GSE2603	U133A	MAS5	99	37	9	58	n.a.	65	27	[22]
Villejuif	GSE7390	U133A	RMA	50	80	26	72	Untreated	108	22	[23]
ExpO	GSE2109	U133A	MAS5	301	31	32	65	n.a.	n.a.	n.a.	[24]
Total				1086	36	34	73		67	236	

sion data as previously described.<sup>27–30</sup> The oestrogen receptor status was determined using Affymetrix probe set 205225\_at and the ErbB2 status using Affymetrix probe set 216836\_s\_at. The cutoff value for ER and ErbB2 positivity in each dataset was determined by fitting two normal distributions<sup>31</sup> on normalised ER and ErbB2 expression data, respectively (see Suppl. Fig. 7). Using the derived cutoff values, a specificity of 88.0% and a sensitivity of 94.4% were observed when the chip-based ER status was compared to immunohistochemical obtained ER status (available for 742 of the 1086 samples), while the specificity and sensitivity of chip-based ErbB2 status were 97.8% and 46.9%, respectively, compared to 3+ staining in immunohistochemistry with HER2 antibody (data available for 290 samples). As a surrogate marker for cellular proliferation we used the expression of the proliferation marker Ki67 (ProbeSets 212020-212023\_s\_at). The distribution of Ki67 expression values is not bimodal as those of ER and ErbB2 presumably because it corresponds to the proportion of Ki67 positive proliferating cells in the sample. Appropriate cutoff values that distinguish between high and low proliferative activities in a clinically relevant manner using Ki67 immunohistochemistry in breast cancer have not been universally established.<sup>32</sup> Thus, a conservative median split according to Ki67 gene expression was applied, which corresponds to a percentage of MIB-1 positive cells of 16–17%.<sup>33</sup> As the second method to determine the proliferative state of the tumour we used a series of cell cycle-associated genes recently described as 'genomic grade index (GGI)'.<sup>20,34</sup> Plexin B1 expression values are based on ProbeSet 215807\_s\_at on the Affymetrix U133A microarray whose measurements were poor in some datasets (e.g. the Rotterdam dataset,<sup>35</sup> see Suppl. Fig. 8). As a quality control, we used the correlation of Plexin B1 expression with the ER status of the samples (an exploratory analysis had revealed a significant higher Plexin B1 expression in ER positive cancers in line with our previous analyses<sup>14</sup>). To allow comparison of Plexin B1 expression between different datasets we used a median split of each dataset according to Plexin B1 Affymetrix data (ProbeSet 215807\_s\_at). Moreover, to avoid the confounding effect by the ER status on Plexin B1 expression in further analyses only ER positive tumour samples were used (see Section 3). For these analyses, the median split of Plexin B1 was applied only to the ER positive subgroup to prevent a confounding effect of the relative proportions of ER positive and negative tumours in the different datasets.

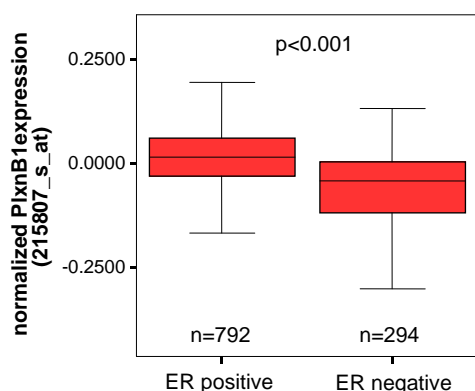
### 2.3. Statistical analysis

All the reported *p*-values are two sided, and *p*-values of less than 0.05 were considered to indicate a significant result. Subjects with missing values were excluded from the analyses.  $\chi^2$ -Test was used to test for associations between Plexin B1 expression of tumours and categorical parameters. For use as a binary variable Affymetrix mRNA expression data of Plexin B1 were categorised using a median split (see above). Although it is also possible to use Plexin B1 expression as a continuous prognostic factor, it is more appropriate and practical to group the tumours into two risk categories allowing, e.g. a direct comparison of Kaplan–Meier curves between the groups.<sup>14</sup> The results of the analyses did not change sub-

stantially when Plexin B1 levels were used in a continuous fashion. Survival intervals were measured from the time of surgery to the time of death from disease or of the first clinical or radiographic evidence of disease recurrence. Data for women in whom the envisaged end-point was not reached were censored as of the last follow-up date or at 120 months. We constructed Kaplan–Meier curves and used the log rank test to determine the univariate significance of the variables. A Cox proportional-hazards regression model was used to



**Fig. 1 – Prognostic value of Plexin B1 expression for disease-free survival. A median split in the full cohort was applied to stratify samples into groups with high and low Plexin B1 expression. Kaplan–Meier analysis of disease-free survival of all 768 patients with the available follow-up is presented in (A). In addition, results for patients with ER positive and ER negative tumours are separately given in (B) and (C), respectively.**



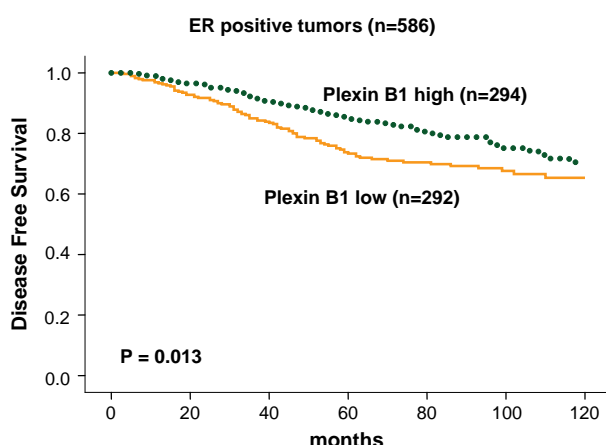
**Fig. 2 – Correlation of Plexin B1 expression and ER status of the tumour.** Box plots of normalised Plexin B1 expression values are given separately for ER positive and negative tumour samples ( $n = 1086$ ).

examine simultaneously the effects of multiple covariates on survival. The effect of each variable was assessed with the use of the Wald test and was described by the hazard ratio, with a 95% confidence interval. The model included age, tumour size, lymph node status, histological grading, ErbB2, Ki67 as well as Plexin B1 expression. All analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL).

### 3. Results

#### 3.1. Prognosis of breast cancers according to the expression of Plexin B1 in a pooled analysis

For survival analysis of combined datasets ( $n = 768$  samples with follow-up data), a median split of expression values of



**Fig. 3 – Prognosis of ER positive tumours stratified by median expression of Plexin B1 within this subgroup.** A median split in the subcohort of ER positive cancers was applied to stratify samples into groups with high and low Plexin B1 expression. Kaplan-Meier analysis of disease-free survival of the 586 patients with ER positive tumours and the available follow-up is shown.

Plexin B1 was used. There was a significant difference in survival favouring tumours with a high Plexin B1 expression (log rank  $p < 0.001$ , Fig. 1A). However, when stratifying data according to ER status, the prognostic value was confined to ER positive cancers (ER positive: log rank  $p = 0.024$ ; ER negative: log rank  $p = 0.85$ , Fig. 1B and C). On the other hand, as shown in Fig. 2 the analysis of Plexin B1 expression values according to the ER status of the tumour revealed a significant higher expression of Plexin B1 in ER positive tumours ( $p < 0.001$ , Mann-Whitney) in line with our previous observations.<sup>14</sup> Thus, in subsequent analyses we included only ER positive tumours to avoid confounding effects caused by the correlation of Plexin B1 expression and ER status and the different prognosis of ER negative and ER positive subtypes of breast cancer. For these analyses, the median split of Plexin B1 was applied only to the ER positive subgroup to prevent a confounding effect of the relative proportions of ER positive and negative tumours in the different datasets. This median split of Plexin B1 expression among ER positive breast cancers also revealed a significant better disease-free survival of patients with high Plexin B1 expression (log rank  $p = 0.013$ , Fig. 3).

#### 3.2. Correlation of Plexin B1 expression with the clinical characteristics of ER positive breast cancers and previously described subtypes

The clinical characteristics of  $n = 792$  ER positive breast cancers stratified by high and low Plexin B1 expression are given in Table 2. No significant difference according to tumour size ( $\chi^2$ -test,  $p = 0.44$ ), nodal status ( $p = 0.28$ ) and age ( $p = 0.93$ ) was observed between the two groups. A trend for an association between Plexin B1 low expressing tumours and poor histological grading was detected ( $p = 0.061$ ). Furthermore, the group of tumours with a low Plexin B1 expression showed a significant higher proportion of ErbB2 positive ( $p = 0.05$ ) and high proliferating cancers as measured by Ki67 expression ( $p = 0.016$ ).

Our observations that Plexin B1 is positively correlated with ER status but inversely correlated with proliferation might suggest that the loss of Plexin B1 is a surrogate marker for the Luminal B subgroup of breast cancers. Recently, it has been demonstrated that the intrinsic subtype of Luminal B according to Sorlie et al.<sup>36</sup> is best defined as ER positive with a high proliferation.<sup>21</sup> A further publication thoroughly demonstrated the application of the intrinsic subtype system for Affymetrix datasets.<sup>37</sup> We used these data to analyse the correlation of Plexin B1 expression with the Luminal B subtype. As shown in Fig. 4A we observed a significant higher Ki67 expression as expected in the Luminal B compared to the Luminal A subgroup. In contrast, no difference in Plexin B1 expression between Luminal A and Luminal B tumours was observed. Furthermore, we analysed the correlation of Plexin B1 expression and proliferation among ER positive tumours in more detail. We used both Ki67 expression and the 'genomic grade index (GGI)'<sup>20</sup> which represents a cluster of proliferation-associated genes. As shown in Fig. 4B this analysis also demonstrated that there is no simple relationship of Plexin B1 expression and proliferation in ER positive tumours.

**Table 2 – Clinical characteristics of ER positive breast cancers according to high and low Plexin B1 expression.**

		Plexin B1 low <sup>a</sup>	Plexin B1 high <sup>a</sup>	p-Value
		n = 393 (49.6%)	n = 399 (50.4%)	
Tumour size <sup>b</sup>	<2 cm	99 (16.7%)	113 (19.1%)	0.44
	>2 cm	191 (32.3%)	189 (31.9%)	
Nodal status <sup>c</sup>	Node negative	165 (28.2%)	160 (27.3%)	0.28
	Node positive	120 (20.5%)	141 (24.1%)	
Grading <sup>d</sup>	Grade1 and 2	197 (32.8%)	228 (37.9%)	0.061
	Grade 3	97 (16.1%)	79 (13.1%)	
Age <sup>e</sup>	<50	108 (16.7%)	110 (17.0%)	0.93
	>50	214 (33.1%)	214 (33.1%)	
ErbB2	ErbB2 negative	348 (43.9%)	370 (51.5%)	0.050
	ErbB2 positive	45 (5.7%)	29 (3.7%)	
Ki67	Ki67 low	178 (22.5%)	215 (27.1%)	0.016
	Ki67 high	215 (27.1%)	184 (23.2%)	

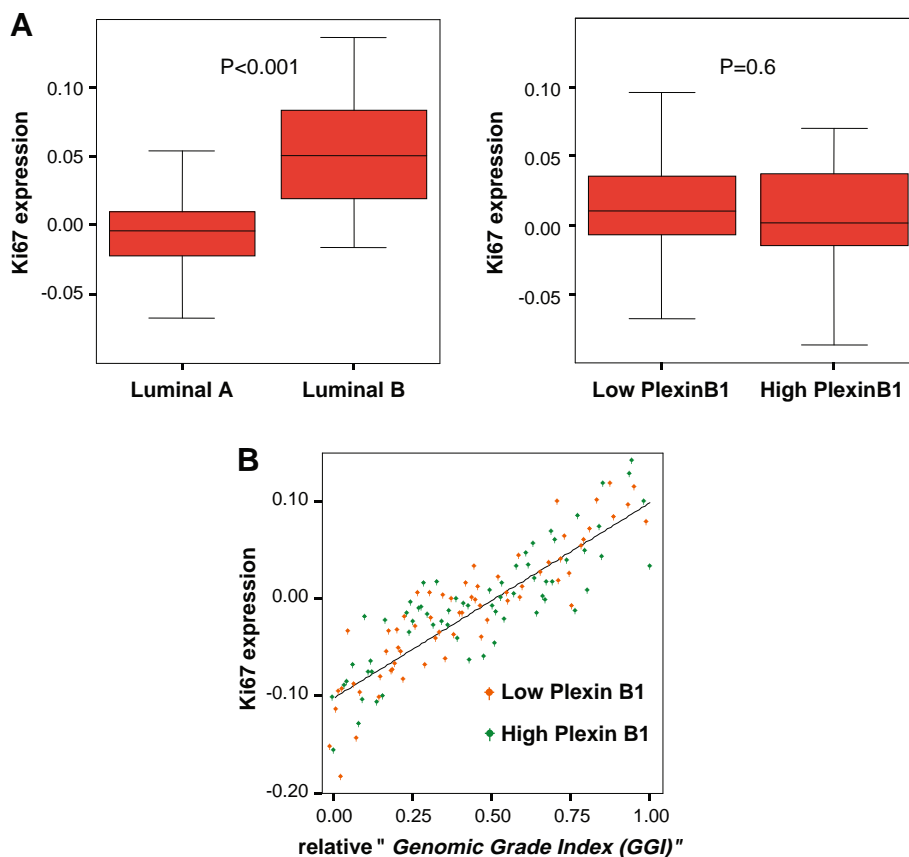
a Samples were stratified according to median Plexin B1 expression among ER positive tumours only.

b Information on tumour size was not available for n = 200 patients.

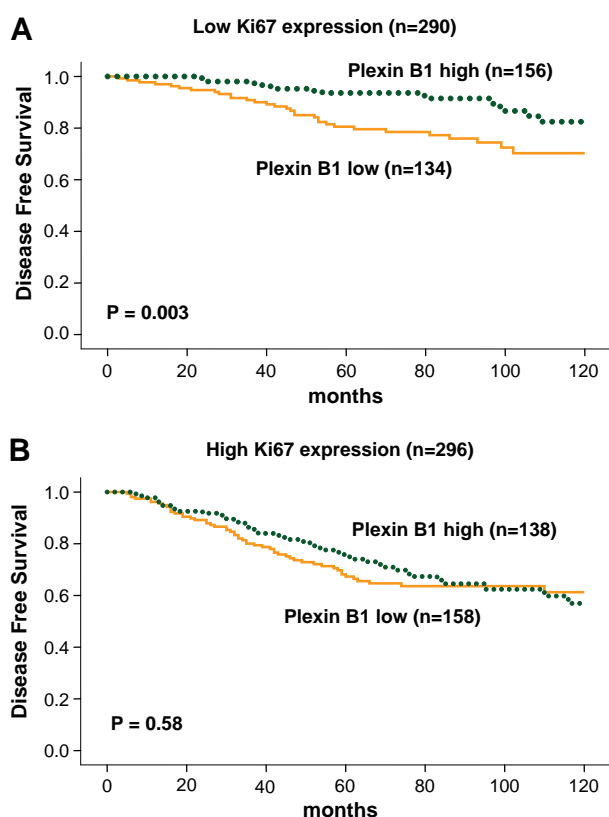
c Information on nodal status was not available for n = 206 patients.

d Information on tumour grade was not available for n = 191 patients.

e Information on age was not available for n = 146.



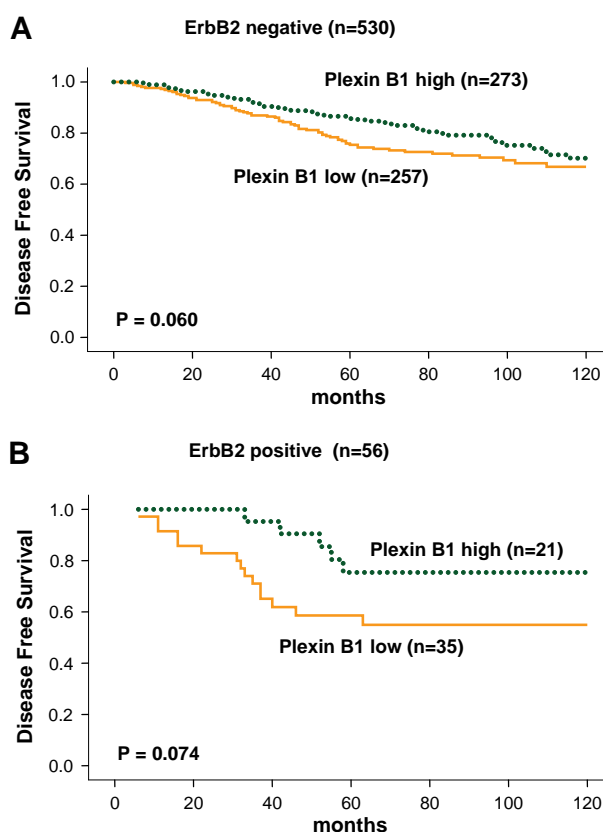
**Fig. 4 – Relationship of Plexin B1 expression, Luminal B subtype and proliferation among ER positive tumours. (A) Luminal A and B subtypes of ER positive tumours differ in their expression of Ki67 (left side) while ER positive tumours with low and high Plexin B1 expression do not (right side). The Stockholm dataset was used for this analysis where the intrinsic subtypes have been precisely mapped.<sup>37</sup> (B) A series of proliferation markers as combined in the genomic grade index (GGI)<sup>20</sup> correlate with Ki67 expression but show no straight relationship to Plexin B1 expression among ER positive tumours.**



**Fig. 5 – Prognostic value of Plexin B1 expression for disease-free survival in the subgroups of ER positive tumours with low and high proliferation.** ER positive tumours were stratified into samples displaying low (A) and high (B) proliferation using median Ki67 expression as a surrogate marker. The prognostic value of Plexin B1 expression in the two subgroups is separately presented in panels A and B. High and low Plexin B1 refers to the median split among ER positive samples as in Fig. 3.

**3.3. Prognostic value of Plexin B1 expression among ER positive breast cancers stratified by the proliferative state of the tumour**

We stratified ER positive samples according to the expression of Ki67 as a surrogate marker for the proliferative status of the tumour. A median split of Ki67 expression was performed among ER positive samples. Subsequently, the prognostic value of Plexin B1 expression was analysed in the respective subgroups. As shown in Fig. 5A, the prognosis of tumours with high and low Plexin B1 expression differed clearly among those ER positive samples with low proliferation (5 year DFS  $93.6 \pm 2.1\%$  versus  $80.5 \pm 3.6\%$  for high Plexin B1 versus low Plexin B1, respectively;  $p = 0.003$ ) resulting in a hazard ratio of 2.45 (CI 1.34–4.47,  $p = 0.004$ ). In contrast, the prognostic value of Plexin B1 expression was not significant in the subgroup of patients with ER positive tumours characterised by high proliferation (5 year DFS  $74.9 \pm 3.9\%$  versus  $67.3 \pm 3.9\%$  for high Plexin B1 versus low Plexin B1, respectively;  $p = 0.58$ ).



**Fig. 6 – Prognostic value of Plexin B1 expression for disease-free survival in ER positive tumours with a negative and positive ErbB2 status, respectively.** ER positive tumours were stratified into samples with a negative (A) and positive (B) ErbB2 status, respectively. The prognostic value of Plexin B1 expression in the two subgroups is separately presented in panels A and B. High and low Plexin B1 refers to the median split among ER positive samples as in Figs. 3 and 4.

**3.4. Prognosis of ER positive breast cancers according to the expression of Plexin B1 stratified by ErbB2 status**

Stratification of ER positive breast cancers by both ErbB2 status and Plexin B1 expression revealed a trend to a better prognosis for a high Plexin B1 expression in both ErbB2 negative and positive tumours as shown in Fig. 6. The 5-year disease-free survival among patients with ErbB2 negative tumours was  $85.6 \pm 2.2\%$  and  $75.4 \pm 2.8\%$  for high and low Plexin B1 expression ( $p = 0.060$ , Fig. 6A), respectively. Patients with ErbB2 positive tumours displayed 5-year DFS rates of  $75.4 \pm 9.6\%$  and  $58.6 \pm 8.5\%$  for high and low Plexin B1 expression ( $p = 0.074$ , Fig. 6B), respectively. Thus, the prognostic value of Plexin B1 may be higher for ErbB2 positive tumours while the number of samples is too small to reach significance (Fig. 6B).

**3.5. Multivariate Cox regression analysis**

A multivariate Cox regression analysis was performed on  $n = 349$  ER positive patients for which data on all standard

parameters (tumour size, lymph node status, grading, age, ErbB2 and Ki67 expression) were available and the results are presented in Table 3. Analysis of these standard parameters and Plexin B1 expression in relation to disease-free survival revealed that Plexin B1 (HR 1.59, 95% CI 1.03–2.47,  $p = 0.04$ ) remained a significant prognostic marker besides tumour size (HR 2.27, 95% CI 1.33–3.89,  $p = 0.003$ ) and Ki67 (HR 1.78, 95% CI 1.12–2.84,  $p = 0.02$ ). Since in univariate analysis the prognostic value of Plexin B1 was restricted to those ER positive tumours with low proliferation, we performed an additional Cox regression analysis for those  $n = 175$  patients. As presented in Table 3 only Plexin B1 (HR 2.1, 95% CI 1.0–4.5,  $p = 0.05$ ) and tumours size (HR 2.7, 95% CI 1.2–6.3,  $p = 0.02$ ) displayed a significant result among those patients.

#### 4. Discussion

This large scale expression analysis of Plexin B1 measured by microarray analysis aimed to validate our recently published findings on the prognostic value of Plexin B1<sup>14</sup> in breast cancer patients. The analysis verified the previous results that Plexin B1 is an ER-dependent marker. In our previous study, we found a strong prognostic value of Plexin B1 even though a proportion of 33.6% of ER negative breast cancer patients was present in the former cohort. Here, we could demonstrate that Plexin B1 is of prognostic relevance only for ER positive breast cancers (using both median splits of Plexin B1 expression for all samples as well as for ER positive cancers only). The loss of Plexin B1 expression in ER positive breast cancers is associated with poor pathohistological grading, ErbB2 overexpression and a high proliferative state of tumours, confirming our previous data. However, when first stratifying ER positive tumours according to Ki67 expression a significant prognostic value of Plexin B1 was only observed for the low proliferating subgroup (Fig. 5), suggesting that Plexin B1 might be a candidate marker for the identification of patients with an elevated risk of recurrence but characterised as low risk patients by standard parameters (ER positive with low grade). In addition, the difference in disease-free survival according to Plexin B1 expression seems to be more pronounced in the subgroup of ErbB2 positive ER positive tumours (Fig. 6B). While this trend is not yet significant due to the small number of samples in this subgroup ( $n = 56$ ) it might suggest a possible influence of Plexin B1 on the ErbB2 pathway. This might be especially interesting since *in vitro* studies have shown that Plexin B1 couples with ErbB2 in signal transduction.<sup>13</sup> On the other hand, there are considerable data on Plexin B1 suggesting that this receptor is involved in cellular adhesion, cell-cell dissociation and invasive growth,<sup>3</sup> while its precise biological function in breast cancer is not yet clear. Loss of the tumour suppressor function of Plexin B1 could enhance the deregulation of tumour cell adhesion and increase migration.<sup>10–12</sup> The presence of Plexin B1 can also sequester its ligand Sema4D. Since Sema4D has a proangiogenic function on endothelial cells we thus had alternatively speculated that the loss of Plexin might promote angiogenesis of the tumour.<sup>14</sup> Recent work on microarray profiling of breast cancers demonstrated that there might

**Table 3 – Cox regression analysis of standard parameters and Plexin B1 expression in relation to disease-free survival in patients with ER positive breast cancers.**

Parameter	All ER positive tumours				Subgroup of low proliferating ER positive tumours					
	$n_1^a$	$n_2^a$	p-Value <sup>b</sup>	HR	(95% confidence interval (CI))	$n_1^a$	$n_2^a$	p-Value <sup>b</sup>	HR	(95% CI)
Plexin B1	170	179	0.04	1.59	(1.03–2.47)	75	100	0.05	2.1	(1.0–4.5)
Lymph node status	212	137	0.62	1.13	(0.70–1.81)	113	62	0.76	0.9	(0.4–2.0)
Age	213	136	0.96	0.99	(0.62–1.59)	110	65	0.22	0.6	(0.3–1.3)
Pathological grading	94	255	0.77	1.07	(0.67–1.71)	26	149	0.83	1.1	(0.4–2.9)
Tumour size	215	134	0.003	2.27	(1.33–3.89)	92	83	0.02	2.7	(1.2–6.3)
ErbB2 status	32	317	0.14	1.57	(0.86–2.84)	11	164	0.30	1.8	(0.6–5.7)
Ki67	174	175	0.02	1.78	(1.12–2.84)	–	–	–	–	–

a Data on all parameters were available for  $n = 349$  patients with ER positive tumours ( $n = 175$  in the low proliferating subgroup).

b Significant p-values are given in bold.

be only a few major determinants discriminating molecular breast cancer subtypes.<sup>36,38</sup> Besides the main distinction of ER positive and negative tumours types as well as HER2, it has been suggested that the proliferation status of the tumour is the far most important characteristic.<sup>20,34</sup> Proliferation seems to be the main distinction of the Luminal A and Luminal B 'intrinsic subtypes'<sup>21</sup> and it was proposed as the main driving force behind most prognostic gene signatures.<sup>39</sup> However, it seems clear that alterations in cellular adhesion should immediately result in changes in proliferation. Thus, a correlation with the proliferation status could also be a confounding epiphenomenon. Regarding Plexin B1 expression we could show that although its loss is associated with a higher proliferation it is not just a surrogate marker for high proliferating ER positive tumours and the Luminal B intrinsic subtype.

In multivariate analysis, Plexin B1 remained a significant factor besides tumour size and Ki67 expression in ER positive patients. However, it should be noted that unexpectedly neither lymph node status nor histological grading and ErbB2 revealed a significant result in this analysis. In univariate analyses, both tumour grade and ErbB2 were significant in the sample cohort but lymph node status was not (see Suppl. Table 4). The smaller number of cases in the multivariate analysis could have contributed to the loss of significance of tumour grade and ErbB2. However, an overlap of the prognostic information of tumour grade and ErbB2 with those of Ki67 or Plexin B1 might also be the reason for this effect. We observed a higher risk for patients with ER positive tumours with low Plexin B1 expression during the first five years with the maximal hazard ratio at year 3–4 after diagnosis (see Suppl. Fig. 9). This is reminiscent to the well-known difference between ER negative and ER positive tumours.<sup>40,41</sup> However, the maximal HR for ER negative tumours was observed already after the first year and drops sharply thereafter (Suppl. Fig. 9).

Our dataset was still too small for a thorough analysis of a potential predictive value of Plexin B1 for treatment. We observed a benefit for Plexin B1 expression in both subgroups of patients with either endocrine or cytotoxic treatment but no significant difference in the group of untreated patients (data not shown). However, the small sample size and different characteristics of the subgroups did not allow a sound conclusion.

Thus, our data so far suggest that Plexin B1 might be a valuable new prognostic marker in ER positive breast cancer and could be helpful in risk assessment of those patients. To incorporate Plexin B1 as a new prognostic tool for the use in clinical routine, an immunohistochemical testing would be necessary. In our previous work, we could demonstrate that expression analysis can be validated by means of PCR, as well as immunohistochemistry.<sup>14</sup> However, a further study validating different antibodies and scoring systems would be indispensable before evaluating this marker in a prospective clinical trial.

In conclusion, Plexin B1 is a strong prognostic marker in ER positive breast cancer. In this large scale analysis, we could verify our initial observations but further investigations are needed to validate Plexin B1 as a prognostic tool according to international guidelines.

## Conflict of interest statement

None declared.

## Role of the funding source

The funding sources were not involved in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

## Acknowledgements

We thank Samira Adel and Katherina Kourtis for an expert technical assistance. This work was supported by grants from the Deutsche Krebshilfe, the Margarete Bonifer-Stiftung, Bad Soden, the BANSS-Stiftung, Biedenkopf, and the Dr. Robert Pflieger-Stiftung, Bamberg.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ejca.2008.10.016](https://doi.org/10.1016/j.ejca.2008.10.016).

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