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PRECLINICAL STUDY

Overexpression of the cancer stem cell marker CD133 confers a poor prognosis in invasive breast cancer

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Abstract

Purpose CD133/ prominin 1 is a cancer stem cell marker associated with cancer progression and patient outcome in a variety of solid tumours, but its role in invasive breast cancer (BC) remains obscure. The current study aims to assess the prognostic value of CD133 expression in early invasive BC.

Methods *CD133* mRNA was assessed in the METABRIC cohort and at the proteomic level using immunohistochemistry utilising a large well-characterised BC cohort. Association with clinicopathological characteristics, expression of other stem cell markers and patient outcome were evaluated.

Results High expression of CD133 either in mRNA or protein levels was associated with characteristics of poor prognosis including high tumour grade, larger tumour size, high Nottingham Prognostic Index, HER2 positivity and hormonal receptor negativity (all; $p < 0.001$). High CD133 expression was positively associated with proliferation biomarkers including p16, Cyclin E and Ki67 ($p < 0.01$). Tumours expressing CD133 showed higher expression of other stem cell markers including CD24, CD44, SOX10, ALDHA3 and ITGA6. High expression of CD133 protein was associated with shorter BC-specific survival ($p = 0.026$). Multivariate analysis revealed that CD133 protein expression was an independent risk factor for shorter BC-specific survival ($p = 0.038$).

Conclusion This study provides evidence for the prognostic value of CD133 in invasive BC. A strong positive association of BC stem cell markers is observed at the protein level. Further studies to assess the value of stem cell markers individually or in combination in BC is warranted.

Keywords Cancer Stem Cell · Invasive breast cancer · Prognosis · CD133

Abbreviations

BC	Breast cancer
BCSS	BC-specific survival
CI	Confidence intervals
ER	Oestrogen
HR	Hazard ratio
HER2	Human epidermal growth factor receptor 2
METABRIC	Molecular taxonomy of breast cancer international consortium

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NPI	Nottingham Prognostic Index
PR	Progesterone
TCGA	The cancer genome atlas

Introduction

Breast Cancer (BC) is a heterogeneous disease with significant clinical, pathological and molecular diversities between tumours. Despite improvements in early detection and management of BC, predicting outcomes remains imprecise, and for this reason many ongoing studies are now seeking to identify novel prognostic and predictive targets to improve BC treatment decision making. Cancer stem cells (CSCs) are believed to have a role in self-renewal, differentiation and carcinogenesis of a variety of malignancies [1]. Breast CSCs are proposed to have a key role in primary tumourigenesis and may also contribute to tumour heterogeneity [2, 3]. In vitro studies using *BRCA*1-associated BC cell lines contain $CD44^{+}/CD24^{low}$ and $CD133^{+}$ cells, displayed CSC properties such as elevated rate of proliferation and tumour forming capability [4]. Evaluation of such CSC markers in BC at the protein levels such as ALDH1 and $CD44^{+}/CD24^{low}$ expression showed association with high histological grade, high proliferative activity and oestrogen receptor (ER) negativity [5]. Microarray gene-expression data mining using $CD44^{+}/CD24^{low/-}$ tumour cells against normal breast epithelium resulted in the development of CSC signatures, enabling prediction of distant metastasis-free survival in independent patient datasets [6, 7].

CD133, also known as prominin 1, is a transmembrane protein expressed on hematopoietic stem cells. It is a putative CSC marker [4] with CD133 expressing cells possessing stem cell-like characteristics including self-renewal, high proliferation and drug resistance; substantiating a tumourigenic role of CD133-expressing cells [8]. CD133 expression is also associated with chemotherapy resistance in tumours which might be explained by the relationship between CD133 and the upregulation of anti-apoptotic proteins such as Bcl-2 and Bcl-xL which are associated with higher levels of CD133 + in glioma stem cells [9] and are overexpressed in sub-populations of BC. Similar to neural stem cells (NSCs), CD133-positive CSC showed increased expression levels of CXCR4 which is a critical protein for the adhesion and/or migration of tumour cells, indicating an important role in migration and may play an important role in tumour invasion [10]. CD133 also plays a crucial role in cell differentiation, proliferation, apoptosis and regulation of cancer-related signaling including MAPK, PLC-β2 and Akt pathways [11, 12]. CD133 is a promising candidate for the identification of CSC in BC including aggressive HER2+ and triple-negative classes [13, 14]. Reports have shown the association of $CD133^{+}$CSCs with the extracellular signal-regulated kinase (ERK) signaling pathway [15] and ERK pathways are associated with increased motility, invasion and cancer progression [16]. Based on the aforementioned studies, it was hypothesised that CD133 plays an important role in BC progression and that expression might identify subgroups of BC with stem cell characteristics and poor prognosis. Thus, in this study, we investigated the association between CD133 and clinicopathological factors of BC at the mRNA and protein levels in large invasive early-stage BC cohorts. Because of the association of other CSC proteins with CD133, its association with other BC stem cell markers was also explored.

Materials and methods

Study cohort

CD133 protein expression was evaluated using a well-characterised cohort of early-stage primary invasive BC as previously described [17–19]. Outcome data, breast cancer-specific survival (BCSS) was maintained on a prospective basis. BCSS was defined as the interval in months from the date of primary surgery to the time of death of BC. The clinicopathological parameters for METABRIC and Nottingham series of patients are summarised in Supplementary Table 1.

Genomic and transcriptomic analysis of CD133

The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) [20] ($n = 1980$) was used to evaluate *CD133* mRNA expression. DNA/RNA was isolated from fresh frozen samples and genomic and transcriptional profiling were obtained using the Affymetrix SNP 6.0 and Illumina HT-12v3 platforms, respectively, as described previously [20, 21]. The assessment of the clinicopathological impact of *CD133* transcription and its association with clinical outcome was performed by setting a cut-off point for the mRNA expression of *CD133* at the median. The clinicopathological significance of *CD133* mRNA expression using Breast Cancer Gene-Expression Miner v4.0 (bcGenExMiner v4.0) was performed for external validation [22] as used in other published studies [23, 24]. The Cancer Genome Atlas (TCGA) dataset ($n = 818$) [25] was also used for external validation of *CD133* mRNA expression.

Immunohistochemistry

To examine CD133 expression, a rabbit monoclonal antibody against human CD133 [86,781, Cell signaling, New England Biolabs (UK)] was with verification of specificity initially confirmed by Western blot analysis of whole cell lysates of MCF-7, MDA MB468 and SKBr3 cell lines

obtained from the American Type Culture Collection; Rockville, MD, USA using 1:1000 dilution of the primary antibody, and fluorescent secondary antibodies at (1:15,000) (IR Dye 800CW donkey anti-rabbit and 680RD donkey anti-mouse, LI-COR Biosciences, UK). 5% milk (Marvel Original Dried Skimmed Milk, Premier Food Groups Ltd, St Albans, UK) was used for blocking. Mouse β-Actin (A5441, Sigma-Aldrich; Clone AC-15; Sigma, UK) at 1:5000 was used as a house-keeping protein. A protein ladder (Page Ruler Plus Pre-Stained Protein Ladder, ThermoScientific, Waltham, MA, USA) was included. To visualise bands, fluorescence at wavelengths of 600, 700 and 800 nm was used on a LiCor Odyssey Fc with image studio 4.0 (LI-COR Biosciences). The specificity of the antibody was validated by the presence of a single specific band at the predicted size (97 kDa; Fig. 1a).

For evaluation of the pattern of CD133 protein expression, immunohistochemical (IHC) was initially assessed in ($n = 10$) full-face BC tissue sections. For assessment of its expression in the whole cohort, tumour samples were arrayed onto tissue microarrays (TMAs) as previously described [17]. IHC was performed on tissue sections using the Novolink Max Polymer Detection system (Leica, Newcastle, UK). In brief, 4-µm-thick sections were deparaffinized with xylene and rehydrated through 100% ethanol. Heat-induced retrieval of antigen epitopes was performed in citrate solution (pH 6.0) for 20 min using a microwave oven (Whirlpool JT359 Jet Chef 1000W). CD133 staining was performed with the rabbit monoclonal antibody (86,781, Cell signaling), diluted (1:75) and incubated for overnight at 4 °C. 3–3′ Diaminobenzidine tetrahydrochloride (Novolink DAB substrate buffer plus) was used as the chromogen. Slides were counterstained with Novolink hematoxylin for 6 min, dehydrated and cover slipped.

The modified H-score method was used in assessing IHC staining, taking the staining intensity and percentage positivity into account [26]. Briefly, the percentages of positively stained tumour cells for each of these intensities were subjectively estimated. Staining intensity (0–3) was multiplied by percentage (0–100) and final scores were obtained, giving

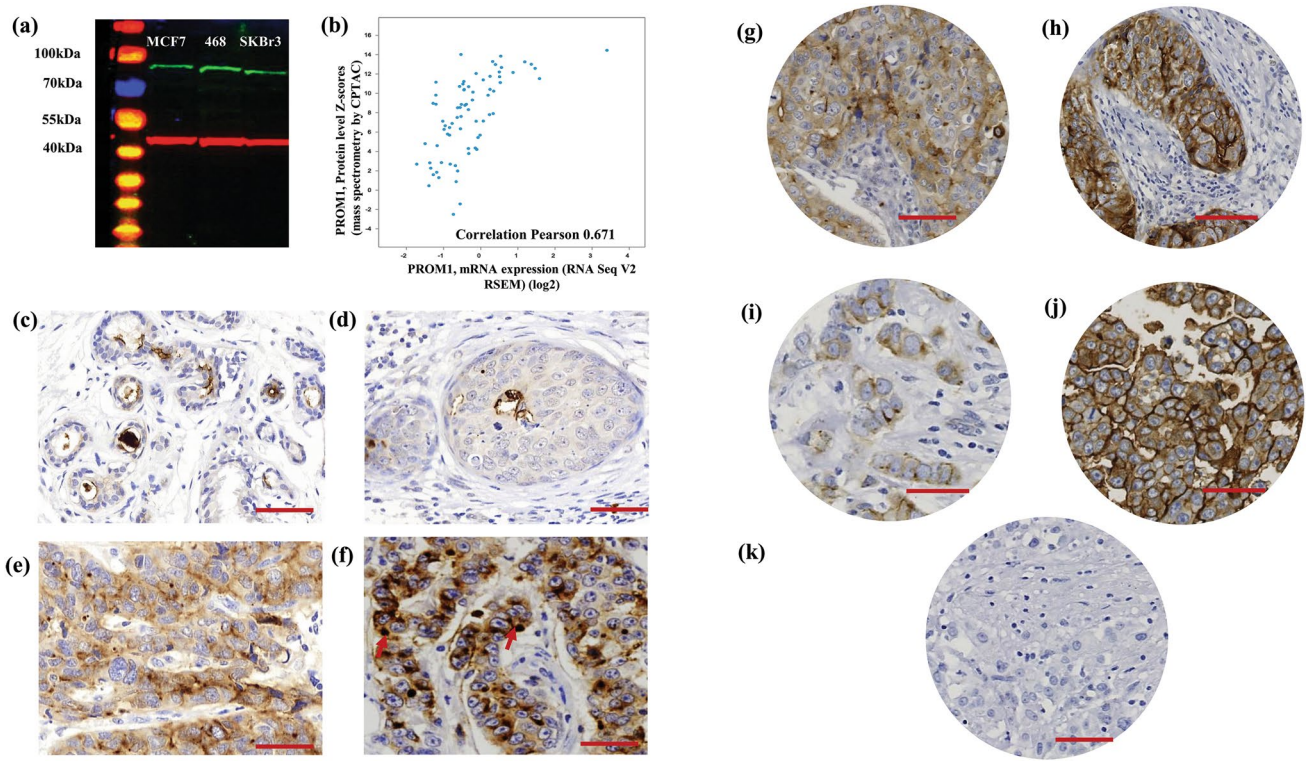

Fig. 1 Western blot and immunohistochemical expression of CD133 in BC. **a** Western blotting results for CD133 expression in MCF7, MDA-MB468 and SKBr3 breast cancer cell lines using rabbit monoclonal antibody against human CD133 [86,781, Cell signaling, New England Biolabs (UK)]. Green and red bands represent CD133 and Beta-Actin (house-keeping), respectively. **b** showing the association between CD133 mRNA and protein (TCGA). Morphological characteristics of CD133 immunohistochemistry in Full-face breast cancer tissue (**c–f**). Normal mammary gland (**c**) and DCIS (**d**) showing absent or weak CD133 staining. **e** showing low immunoreactivity, **f** Showing some area with strong intensity and red arrows represent CD133 staining as accumulated patches/ bubbles within the cytoplasm. CD133 protein expression in breast cancer TMA cores (**g–k**). **g** and **h** showing low and high cytoplasmic (C+) expression, respectively, while **i** and **j** showing low and high membrane (M+) at ×40 magnification, respectively. **k** showing the negative expression in TMA

a range of 0 to 300. High-resolution digital images were generated via scanning the IHC stained slides (Nanozoomer; Hamamatsu Photonics, Welwyn Garden City, UK). Staining was double scored blindly by two researchers including a histopathologist for 25% cores to assess inter-observer concordance. Moreover, the discordant cases were re-scored by the both observers and a final score was agreed. IHC staining and dichotomisation of the other biomarkers included in this study were as per previous publications [18, 27–29]. BC molecular subtypes were defined based on the IHC profile as Luminal A: ER+/HER2− Low Proliferation (Ki67 < 10%), Luminal B: ER+/HER2− High Proliferation (Ki67 ≥ 10%) or ER+/HER2+, HER2-positive class: HER2+ regardless of ER status, Triple Negative (TN): ER−, progesterone receptor (PgR)- and HER2−.

Statistical analysis

IBM SPSS 22.0 (Chicago, IL, USA) software was used for statistical analysis. Univariate analysis was performed using the chi-squared test to evaluate the significance of the association between expression of the biomarkers and the clinicopathological parameters of the data, as well as other previously investigated biomarkers. Spearman's correlation coefficient was carried out to examine the association between two continuous variables. Kaplan–Meier analysis with a log-rank test for significance was performed to assess BCSS. Multivariate Cox Regression analysis with adjustment of covariates was fitted to test independence from standard prognostic factors in BC (nodal stage, tumour grade and tumour size). *P* values were adjusted using Bonferroni correction for multiple testing. A p value of <0.05 was considered significant.

This study was approved by the Nottingham Research Ethics Committee 2 under the title 'Development of a molecular genetic classification of breast cancer'.

Results

CD133 genomic profiling

High *CD133* mRNA expression was associated with high histological grade ($p<0.0001$), triple-negative ($p<0.0001$) and HER2+ ($p=0.004$) subtypes. High *CD133* mRNA expression was associated with negative ER and PgR status, younger age at diagnosis and pre-menopausal women ($p<0.00001$; Table 1). When BC was classified using the intrinsic (PAM50) subtypes, high *CD133* expression was associated with basal-like and HER2+ classes ($p<0.0001$). Similarly, within the METABRIC Integrative Clusters, high *CD133* mRNA expression was associated with clusters 5 (ERBB2 amplified), and 10 (TNBC/basal-like), respectively ($p<0.00001$). In the SCMGENE subtypes, high *CD133* mRNA expression was associated with ER−/HER2− and HER2+ groups ($p<0.00001$). Associations between *CD133* mRNA expression and clinicopathological variables are summarised in Table 1.

Using Breast Cancer Gene-Expression Miner v4.0 high expression of *CD133* mRNA was associated with ER, and PR-negative status ($n=5222$; $p<0.0001$; Supplementary Fig. 1a, b), higher tumour grade ($n=3294$; $p<0.0001$; Supplementary Fig. 1c) and triple-negative status ($n=1191$; $p<0.0001$; Supplementary Fig. 1d). Association of *CD133* mRNA with PAM50 and SCMGENE subtypes was confirmed using the Breast Cancer Gene-Expression Miner v4.0 ($n=5263$; $p<0.0001$; Supplementary Fig. 1e, f). The association of high *CD133* mRNA in our study was in agreement with TCGA data, (Supplementary Fig. 2a–d), younger age at diagnosis, ER− > ER+ and PR− > PR+, where data were available.

When considering expression of other BC stem cell (CSC) biomarker, high *CD133* mRNA showed positive correlation with previously studied CSC (Supplementary Table 2) including *CD24* ($p<0.0001$), *CD44* ($p<0.0001$), *SOX10* ($p<0.0001$), *ALDHA3* ($p<0.0001$), *ITGA6* ($p<0.0001$) and *ALDHA1* ($p=0.003$), while it was negatively correlated with *PTEN* ($p<0.0001$). These associations were confirmed using Breast Cancer Gene-Expression Miner v4.0 (Supplementary Fig. 3a–d).

CD133 protein expression

CD133 protein expression showed a positive correlation with *CD133* mRNA (Spearman's coefficient 0.505; $p<0.00001$), this association was confirmed using TCGA data (Fig. 1b). Full-face tissue sections showed CD133 expression in normal glandular epithelium and DCIS is weak (Fig. 1c, d). In contrast, CD133 immunopositivity was observed in the cytoplasm/membrane of invasive cancer cells, which was stronger compared to the normal epithelial cells (Fig. 1e, f), with some malignant cells featuring accumulation of CD133 expression in granules, patches and bubbles within the cytoplasm of uncertain significance (Fig. 1f; Red arrows).

On TMAs, variable degree of CD133 protein expression in BC is shown in Fig. 1g–k. The H-scores of both cytoplasmic (C+) and membrane (M+) expression did not follow a normal distribution therefore for dichotomisation into low/high expression the median H-score (0) was used, in agreement with previous studies [30]. H-scores for C+ and M+ staining showed a positive correlation (Spearman's coefficient 0.560; $p<0.00001$). Of the 687 informative cores, for CD133 cytoplasmic staining, 31% of tumours showed high expression 69% displayed negative/low expression. For the membrane staining of CD133, 14% displayed high CD133 expression of which 86% showed negative/low expression.

Table 1 Associations between *CD133* mRNA expression and clinicopathological variables in the METABRIC cohort

Clinicopathological Criteria	CD133 mRNA expression		χ^2 and p value
	Negative/low expression *N* (%)	High expression *N* (%)	
Age at diagnosis			
≤ 50	125 (29.5)	299 (70.5)	93.529 (**< 0.00001**)
> 50	864 (55.)	687 (44.3)	
Menopause			
Pre	360 (82.6)	76 (17.4)	29.034 (**< 0.0007**)
Post	1403 (91.5)	130 (8.5)	
Tumour size (cm)			
≤ 2.0	414 (48.3)	444 (51.7)	1.921 (1.72)
> 2.0	566 (51.4)	536 (48.6)	
Histological grade			
1	88 (51.8)	82 (48.2)	23.109 (**0.0001**)
2	433 (56.2)	337 (43.8)	
3	425 (44.6)	527 (55.4)	
Tumour Type			
Ductal	875 (51.4)	826 (48.6)	30.186 (**< 0.0001**)
Lobular	54 (36.7)	93 (63.3)	
Medullary-like	6 (18.8)	26 (81.3)	
Special type	33 (67.3)	16 (32.7)	
Miscellaneous	10 (47.6)	11 (52.4)	
Nottingham prognostic index (NPI)			
Good prognostic group	365 (53.7)	315 (46.3)	5.602 0.366
Moderate prognostic group	529 (48.0)	572 (52.0)	
Poor prognostic group	96 (48.2)	103 (51.8)	
PAM50 subtype			
Luminal A	399 (55.6)	319 (44.4)	446.069 (**< 0.0001**)
Luminal B	398 (81.6)	90 (18.4)	
Basal	47 (14.3)	282 (85.7)	
Her2	105 (43.8)	135 (56.3)	
Normal like	40 (2.1)	159 (79.9)	
IntClustMemb			
IntClustMemb 1	110 (79.1)	29 (20.9)	
IntClustMemb 2	51 (70.8)	21 (29.2)	
IntClustMemb 3	132 (45.5)	158 (54.5)	
IntClustMemb 4	167 (48.7)	176 (51.3)	
IntClustMemb 5	65 (34.2)	125 (65.8)	254.366 (**< 0.00001**)
IntClustMemb 6	61 (71.8)	24 (28.2)	
IntClustMemb 7	132 (69.5)	58 (30.5)	
IntClustMemb 8	171 (57.2)	128 (42.8)	
IntClustMemb 9	71 (48.6)	75 (51.4)	
IntClustMemb 10	30 (13.3)	196 (86.7)	
SCMGENE subtypes			
ER+/HER2− low prolif	204 (55.4)	164 (44.6)	140.111 (**< 0.00001**)
ER+/HER2− high prolif	266 (72.3)	102 (27.7)	
HER2+	38 (34.5)	72 (65.5)	
ER−/HER2−	29 (19.2)	122 (80.8)	
Oestrogen receptor (ER)			
Negative	87 (19.8)	352 (80.2)	207.199 (**< 0.00001**)
Positive	882 (58.9)	616 (41.1)	

Table 1 (continued)

Clinicopathological Criteria	CD133 mRNA expression		χ^2 and p value
	Negative/low expression *N* (%)	High expression *N* (%)	
Progesterone receptor (PR)			
Negative	393 (41.8)	547 (58.2)	48.034 (**< 0.00001**)
Positive	597 (57.4)	443 (42.6)	
Human epidermal growth factor receptor 2 (HER2)			
Negative	896 (51.7)	837 (48.3)	16.102 (**0.0048**)
Positive	94 (38.1)	153 (61.9)	
Triple-negative (TPN) status			
Negative	930 (56.0)	731 (44.0)	165.885 (**< 0.0001**)
Positive	39 (14.1)	237 (85.9)	

Significant p values are highlighted in bold

Similar to the mRNA observation, CD133 protein expression was also associated with poor prognostic parameters. High CD133 C + and M + expression was associated with higher histological grade ($p < 0.0001$), higher mitotic frequency ($p < 0.0001$; Table 2), lower tubule formation ($p = 0.015$) and higher nuclear pleomorphism ($p < 0.0001$). CD133 was highly expressed in triple-negative subtype compared with non-triple-negative BC ($p < 0.0001$; Table 2). Elevated CD133 M + additionally showed association with poor NPI ($p < 0.0001$), large tumour size ($p = 0.004$), younger age at diagnosis ($p = 0.003$) and pre-menopausal status ($p = 0.009$).

High CD133 expression showed significant association with negative ER/PR status ($p < 0.0001$), and positive basal cytokeratins CK5/6, CK14 and CK17 expression (all; $p < 0.0001$; Table 3). The association between basal CKs expression and poor prognosis has been well documented [29]. High expression of CD133 was also positively associated with cell cycle regulators p16 and Cyclin E ($p = 0.0004$ *and* $p < 0.0001$, respectively) and proliferation marker Ki67 ($p < 0.0001$). High CD133 protein was positively associated with EGFR ($p = 0.039$) and PIK3CA ($p = 0.048$; Table 3).

Relationship between CD133 expression and other CSC markers

A significant positive correlation between CD133 C + expression and CD24 (p = *0.005*), CD44 ($p = 0.020$) and SOX10 ($p = 0.017$) was observed. There was a negative association between CD133 C + and PTEN ($p = 0.009$). CD133 M + showed a positive correlation with CD24 ($p < 0.0001$) and CD44 ($p = 0.020$; Supplementary Table 2).

CD133 expression and patient outcome

In the whole METABRIC cohort, though *CD133* mRNA showed a trend of poor patient outcome it did not reach statistical significance. The 10-year BCSS of patients with tumours expressing CD133 C+ or M+ was significantly shorter than that of the low expression subgroup (HR 1.38; $p = 0.026$ and HR = 1.95; $p = 0.0001$; Fig. 2a, b). Combined CD133 cytoplasmic and membrane (CM) survival analysis demonstrated that M + expression was associated with shorter BCSS (HR 1.25; $p < 0.0001$; Fig. 2c). In subgroup analysis, expression of CD133 M+ on its own and in combination with C (±) was predictive of shorter BCSS in the HER2+ subgroup (HR 1.96; $p = 0.04$; HR = 1.29; $p < 0.001$; Fig. 2e–f, respectively). There was no association between CD133 protein and outcome in Luminal A and B or TNBC (Supplementary Fig. 4a–i). In multivariate Cox regression analysis, CD133 M+ [$p = 0.038$, HR 1.4 (95% CI 1.0–2.1; Table 4)] and C+M+ [$p = 0.028$, HR 1.2 (95% CI 1.0–1.3)] protein expression was a predictor of shorter BCSS independent of tumour size [$p = 0.015$, HR 1.4 (95% CI 1.1–2.1)], tumour grade [$p < 0.0001$, HR 2.1 (95% CI 1.6–2.7)] and nodal stage [$p < 0.0001$, HR 2.2 (95% CI 1.8–2.8)] in the whole cohort only.

Discussion

Breast cancer (BC) is a heterogeneous disease with various subtypes [31] differing in terms of morphology, molecular and biological profiles, response to therapy, clinical behavior and outcomes. Over the last decade, there have been numerous studies looking into the role of CD133 as a potential prognostic marker and predictor of patient survival for various cancers including ovarian cancer, glioma and colorectal cancer [32–34]. To our knowledge, however, limited research has been done to evaluate the prognostic roles of CD133 in BC [35]. Thus, the aim of the study was to determine biological and prognostic value of CD133 expression utilising large and well-described BC cohorts. Both transcriptomic and proteomic results revealed that high

Table 2 Clinicopathological associations of CD133 expression in the Nottingham BC series

Clinico pathological criteria	CD133Cytoplasmic staining *N* (%)		χ^2 and *p* value	CD133 membrane staining *N* (%)		χ^2 and *p* value
	Negative/low expression	High expression		Negative/low expression	High expression	
Age at diagnosis						
< 50	159 (66.5)	80 (33.5)	1.673	189 (79.1)	50 (20.9)	14.152
≥ 50	306 (71.3)	123 (28.7)	1.97	382 (89.7)	44 (10.3)	**0.0030**
Menopausal status						
Pre-menopause	173 (67.1)	85 (32.9)	1.389	205 (79.5)	53 (20.5)	14.796
Post-menopause	288 (71.3)	116 (28.7)	3.499	361 (90.0)	40 (10.0)	**0.009**
Histological Grade						
1	74 (74.7)	25 (25.3)	21.463	96 (97.0)	3 (3.0)	57.172
2	167 (79.9)	42 (20.1)		202 (96.7)	7 (3.3)	
3	220 (62.0)	135 (38.0)	< **0.0001**	268 (76.1)	84 (23.9)	< **0.0001**
Tubules						
1	21 (80.8)	5 (19.2)	13.174	24 (92.3)	2 (7.7)	29.044
2	162 (77.5)	47 (22.5)		200 (95.2)	10 (4.8)	
3	254 (64.1)	142 (35.9)	**0.015**	311 (79.1)	82 (20.9)	< **0.0001**
Pleomorphism						
1	11 (91.7)	1 (8.3)	19.479	12 (100.0)	0 (0.0)	**45.772**
2	181 (78.7)	49 (21.3)		227 (97.0)	7 (3.0)	
3	244 (63.0)	143 (37.0)	< **0.0001**	301 (77.6)	87 (22.4)	< **0.0001**
Mitosis						
1	144 (80.0)	36 (20.0)	24.727	176 (96.2)	7 (3.8)	49.827
2	104 (75.9)	33 (24.1)		129 (93.5)	9 (6.5)	
3	189 (60.2)	125 (39.8)	< **0.0001**	237 (75.2)	78 (24.8)	< **0.0001**
IHC subtypes						
ER+/HER2− Low Proliferation	98 (79.7)	25 (20.3)	81.393	120 (96.0)	5 (4.0)	137.761
ER+/HER2− high proliferation	166 (83.4)	33 (16.6)		191 (97.0)	6 (3.0)	
Triple negative	48 (38.4)	77 (61.6)		63 (50.8)	61 (49.2)	
HER2+	69 (63.9)	39 (36.1)	< **0.0001**	89 (83.2)	18 (16.8)	< **0.0001**
Histological type						
Ductal	395 (68.9)	178 (31.1)	8.095	488 (85.5)	83 (14.5)	15.771
Lobular	39 (81.3)	9 (18.7)		46 (97.9)	1 (2.1)	
Medullary-like*	8 (47.1)	9 (52.9)		10 (58.8)	7 (41.2)	
Special type	18 (78.3)	5 (21.7)	0.484	20 (87.0)	3 (13.0)	**0.008**
Stage						
I	283 (68.7)	129 (31.3)	2.603	352 (86.3)	56 (13.7)	1.079
II	142 (70.3)	60 (29.7)		174 (85.3)	30 (14.7)	
III	37 (72.5)	14 (27.5)	3.656	42(84.0)	8 (16.0)	4.692
Tumour size						
≥ 2.0 cm	215 (70.7)	89 (29.3)	0.348	275 (90.2)	30 (9.8)	8.926
< 2.0 cm	247 (68.6)	113 (31.4)	3.672	292 (82.0)	64 (18.0)	**0.004**
Nottingham prognostic index						
Good prognostic group	140 (75.7)	45 (24.3)	4.512	179 (96.8)	6 (3.2)	26.680
Moderate prognostic group	247 (67.1)	121 (32.9)		302 (82.5)	64 (17.5)	
Poor prognostic group	75 (67.6)	36 (32.4)	1.05	86 (78.2)	24 (21.8)	< **0.0001**

Significant *p* values are highlighted in bold

Table 3 Associations of CD133 IHC expression and other BC-related biomarkers within the Nottingham BC series

Clinicopathological criteria	CD133Cytoplasmic staining		χ^2 and p value	CD133 membrane staining (in %)		χ^2 and p value
	Negative/low expression N (%)	High expression N (%)		Negative/low expression N (%)	High expression N (%)	
Oestrogen (ER) status						
Negative	78 (41.7)	109 (58.3)	93.875	114 (61.6)	71 (38.4)	122.601
Positive	382 (80.3)	94 (19.7)	**< 0.0001**	452 (95.2)	23 (4.8)	**< 0.0001**
Progesterone (PR) status						
Negative	149 (54.4)	125 (45.6)	51.920	198 (72.5)	75 (27.5)	68.086
Positive	296 (80.9)	70 (19.1)	**< 0.0001**	349 (95.6)	16 (4.4)	**< 0.0001**
Human epidermal growth factor receptor 2 (HER2)						
Negative	381 (70.6)	159 (29.4)	1.717	464 (86.2)	74 (13.8)	0.624
Positive	70 (64.2)	39 (35.8)	0.210	90 (83.3)	18 (16.7)	0.451
Triple negative						
Negative	408 (77.0)	122 (23.0)	69.726	497 (94.0)	32 (6.0)	150.358
Positive	48 (38.7)	76 (61.3)	**< 0.0001**	63 (51.2)	60 (48.8)	**< 0.0001**
Basal phenotype						
Negative	368 (77.6)	106 (22.4)	53.811	436 (91.8)	39 (8.2)	47.460
Positive	90 (48.4)	96 (51.6)	**< 0.0001**	133 (71.1)	54 (28.9)	**< 0.0001**
Cytokeartin5/6 (CK5/6)						
Negative	328 (76.3)	102 (23.7)	39.204	391 (91.1)	38 (8.9)	78.907
Positive	40 (43.5)	52 (56.5)	**< 0.0001**	50 (54.3)	42 (45.7)	**< 0.0001**
Cytokeratin 14 (CK14)						
Negative	393(72.2)	151 (27.8)	17.552	469 (86.9)	71 (13.1)	4.289
Positive	34 (47.9)	37 (52.1)	**< 0.0001**	56 (77.8)	16 (22.2)	0.073
Cytokeratin 17 (CK17)						
Negative	296 (73.3)	108 (26.7)	18.341	359 (89.1)	44 (10.9)	40.095
Positive	34 (47.9)	37 (52.1)	**< 0.0001**	41 (59.4)	28 (40.6)	**< 0.0001**
Epidermal growth factor receptor (EGFR)						
Negative	373 (72.0)	145 (28.0)	9.856	441 (87.5)	63 (12.5)	6.343
Positive	84 (58.3)	60 (41.7)	**0.0120**	113 (79.6)	29 (20.4)	**0.039**
Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA)						
Negative	89 (75.4)	29 (24.6)	3.741	456 (88.0)	62 (12.0)	6.736
Positive	278 (66.0)	143 (34.0)	0.265	115 (79.9)	29 (20.1)	**0.048**
Ki67						
Negative	150 (73.9)	53 (26.1)	3.388	180 (92.3)	15 (7.7)	15.182
Positive	208 (66.2)	106 (33.8)	0.316	242 (79.3)	63 (20.7)	**< 0.0001**
p16						
Negative	258 (78.2)	72 (21.8)	35.958	301 (90.4)	32 (9.6)	13.123
Positive	112 (536)	97 (46.4)	**< 0.0001**	165 (79.3)	43 (20.7)	**0.0004**
Cyclin E						
Negative	184 (71.9)	72 (28.1)	18.174	238 (93.0)	18 (7.0)	19.570
Positive	34 (45.3)	41 (54.7)	**< 0.0001**	56 (74.7)	19 (25.3)	**< 0.0001**

Significant p values are highlighted in bold

expression of CD133 was significantly associated with poor prognostic characteristics, including high histological grade, younger age, poor NPI, ER−/PgR− tumours and histological subtypes of poor prognosis. This is in agreement with studies of other cancer sites. For example, CD133 is significantly associated with tumour aggressiveness and poor prognosis in ovarian [36], gastric [37] and renal cell carcinomas [38].

CD133 mRNA overexpression was significantly associated with TNBC as previously documented [39]. Within the METABRIC Integrative Clusters, high *CD133* mRNA

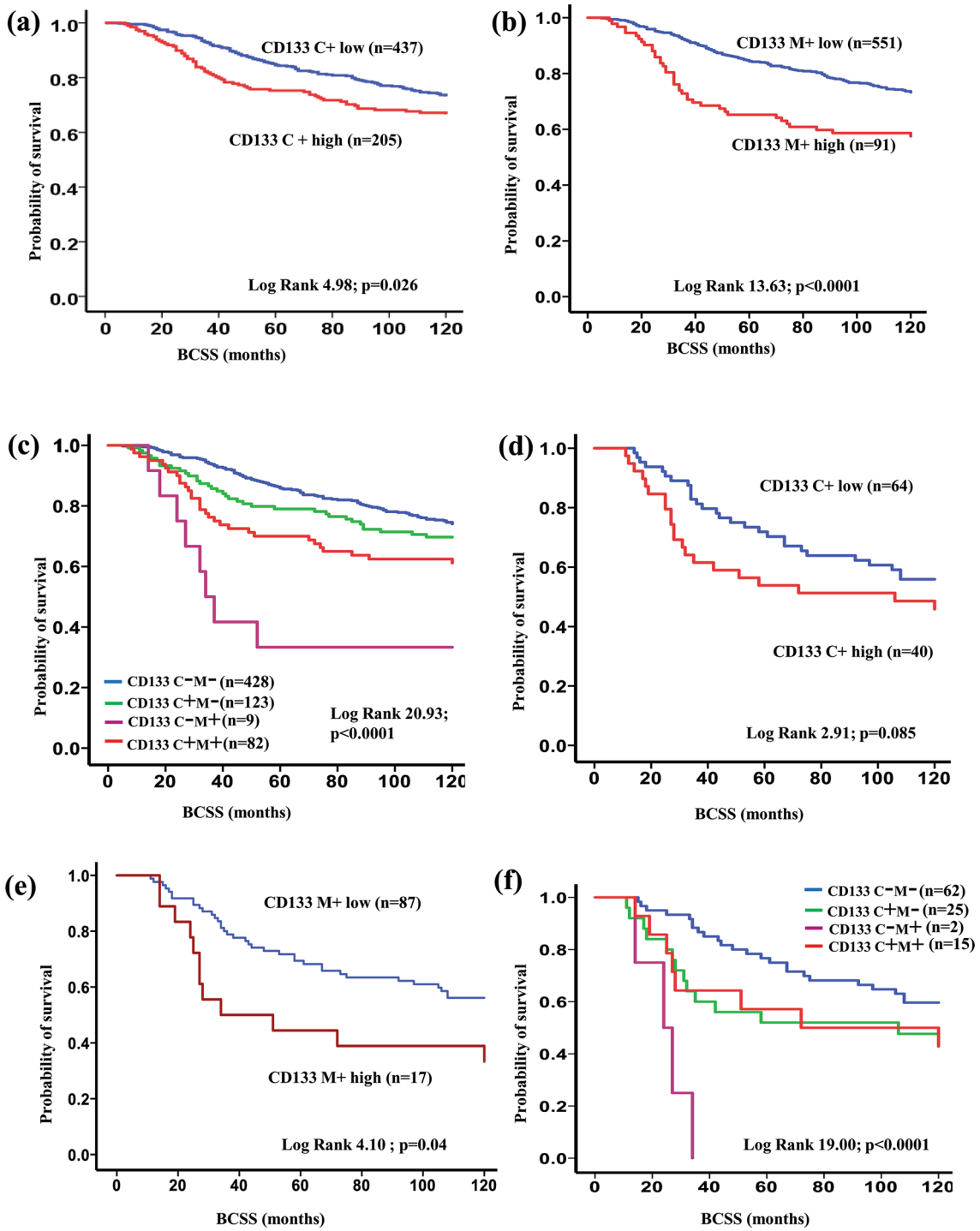

Fig. 2 Kaplan–Meier plots of CD133 protein expression and breast cancer patient outcome. **a** CD133 C+ vs BCSS in whole cohort, **b** CD133 M+ vs BCSS in whole cohort, **c** Combined cytoplasmic and membrane (CM) CD133 expression vs BCSS in whole cohort, **d** CD133 C+ vs BCSS of HER2+ tumours, **e** CD133 M+ vs BCSS of HER2+ tumours and **f** Combined cytoplasmic and membrane (CM) CD133 expression vs HER2+ tumours

expression was associated with clusters 10 (TNBC/basal-like) IC, which provides further support for a potential role in TNBC. TNBC is associated with resistance to chemotherapy and significantly correlated with worse clinical outcome [40]. The expression of CD133 has been negatively associated with β2 isoform of the phosphoinositide-dependent phospholipase C (PLC-β2) [13, 41] and overexpression of PLC-β2 has a role in inducing the conversion

Table 4 Multivariate analysis of prognostic variables and CD133 expression in relation to BCSS in the whole cohort and BC subtypes

Variable	Whole cohort			Luminal A			Luminal B			TNBC			HER2+		
	HR	95%CI	*p* value	HR	95%CI	*p* value	HR	95%CI	*p* value	HR	95%CI	*p* value	HR	95%CI	*p* value
Stage	2.2	1.8–2.8	**< 0.001**	3.3	1.3–3.3	**0.002**	1.8	1.2–2.6	**0.005**	2.0	1.3–3.3	**0.002**	3.2	2.1–5.0	**< 0.001**
Grade	2.1	1.6–2.8	**< 0.001**	1.7	0.5–22.9	0.226	1.9	1.2–3.2	**0.009**	3.3	0.5–22.9	0.226	2.0	0.9–4.2	0.073
Tumour size	1.4	1.1–2.1	**0.015**	1.4	1.1–5.5	**0.034**	1.3	0.7–2.4	0.349	2.4	1.1–5.5	**0.034**	1.3	0.7–2.5	0.335
CD133 C+	1.3	0.9–1.8	0.072	1.5	0.5–1.9	0.942	1.0	0.5–2.1	0.993	0.9	0.5–1.9	0.942	1.4	0.8–2.6	0.221
Stage	2.2	1.8–2.8	**< 0.001**	3.5	1.5–8.5	**0.005**	1.8	1.25–2.6	**0.004**	2.0	1.3–3.2	**0.002**	2.0	1.3–3.2	0.002
Grade	2.1	1.6–2.8	**< 0.001**	1.7	0.7–4.2	0.249	2.0	1.2–3.2	**0.009**	2.8	0.5–19.1	0.295	2.8	0.4–19.2	0.295
Tumour size	1.5	1.1–2.1	**0.016**	1.4	0.4–5.4	0.617	1.3	0.8–2.3	0.372	2.4	1.1–5.6	**0.029**	2.5	1.1–5.6	0.029
CD133 M+	1.4	1.0–2.1	**0.038**	2.0	0.3–16.1	0.507	0.5	0.7–3.8	0.510	1.5	0.8–2.9	0.175	1.5	0.8–2.9	0.175
Stage	2.3	1.8–2.8	**< 0.001**	3.4	1.4–8.0	**0.006**	1.8	1.2–2.6	**0.004**	2.0	1.3–3.2	**0.002**	3.2	2.1–5.0	**< 0.001**
Grade	2.1	1.6–2.7	**< 0.001**	1.7	0.7–4.3	0.243	2.0	1.2–3.2	**0.011**	2.9	0.4–19.5	0.284	2.0	0.9–4.1	0.079
Tumour size	1.5	1.1–2.1	**0.015**	1.4	0.4–5.5	0.616	1.3	0.7–2.4	0.372	2.4	1.1–5.5	**0.034**	1.2	0.7–2.3	0.501
CD133 C+M+	1.2	1.0–1.3	**0.028**	1.3	0.7–2.7	0.409	0.9	0.5–1.5	0.674	1.1	0.9–1.4	0.317	1.2	0.9–1.6	0.103

Significant *p* values are highlighted in bold

of CD133 + cells to $CD133^-$ cells in TNBC resulting in decrease in proliferation and invasion [42]. This implies that CD133 plays a role in tumourigenic pathways and could be a marker of poor prognosis in TNBC.

High CD133 expression was also associated with an increase in cell cycle and proliferation activity indicated by correlation with high expression of Cyclin E1, p16 and Ki67. The proliferation-associated factor p16 is linked with ER-negative BCs and poor patient outcome [43]. The G1–S-phase transition of the cell cycle is associated with both cyclin E and p16 [44]. The role of Cyclin E1-activated cyclin-dependent kinases (CDKs) in the proliferation of cancer stem cells is well documented [45]. Proliferation and differentiation are under control of the cell cycle and the positive association with above markers strengthens the putative role of CD133 in tumour progression.

PI3K signaling plays a crucial role in cell cycle progression, growth and survival [46], and acts as an important mediator of self-renewal/expansion in cancer stem cells [47, 48]. The PI3K pathway is regulated by EGFR, and the overexpression of EGFR in basal-like BC is activated by aberrant PI3K activity. This suggests a positive feedback loop of co-regulation. Evidence from the TCGA breast cancer study suggests that the basal-like BC subtype has the highest level of PI3K activity [25]. We observed that CD133 overexpression was positively associated with EGFR and thereby upregulating PI3K signaling. This implies that CD133 could have a role in basal-like tumourigenic pathways. Clinical trials of EGFR inhibitors highlight the need for predictive biomarkers [49] to select patients. Understanding the functional role of CD133 in regulating the EGFR/PI3K pathway in BC may have clinical application in selecting the right combination of targeted therapies, to maximise efficacy for the individual patient.

CD133 mRNA and protein expression revealed significant positive association with other key CSC markers such CD44, CD24 and SOX10. High CD44 and CD24 expression were associated with aggressive histological features and metastasis [50, 51]. The association of both of these cell surface glycoproteins with CD133 provides further support for CD133 as a marker of adverse prognosis in BC. SOX10 is a transcription factor that regulates the differentiation of neural crest cells [45]. Like CD133, SOX10 expression is associated with tumourigenicity and proliferation in melanoma [52]. Negative correlation between CD133 and the PTEN tumour suppressor was found in our study. *PTEN* loss confers increased self-renewal capacity, resulting in the development of CSC and eventually tumourigenesis. PTEN is one of the most recurrently lost or mutated tumour suppressor genes. Overexpression of PTEN significantly decreases the levels of both CD133 mRNA and protein in glioblastoma [53, 54]. Thus, in summary, evidence suggests that increased CD133 expression is associated with CSC markers and loss of a key tumour suppressor gene.

This study reveals that CD133 is associated with poor prognostic characteristics and short-term survival outcomes in BC, which is in agreement with a previous study [55]. Although CD133 C + and CD133 M + were both linked to shorter BCSS in the whole cohort, membrane positivity only confers the worst patient outcome. Furthermore, high CD133 membrane expression was significantly associated with younger age at diagnosis and pre-menopausal status. Positive association of CD133 and elevated levels of basal cytokeratin confers poor prognosis. CD133 mRNA and protein were also highly expressed in TNBC and HER2+, in concordance with a recent study [39]. Basal cytokeratins are strongly associated with high histological grade (III), ER−, PR− status and worse patient outcome [29]. In the whole

BC cohort, CD133 M + and C + M + protein expression independently predicted short BCSS. Among subgroups, overexpression of CD133 protein appears to play a particularly significant role in HER2+ BC. However, due to the relatively small sample size of the HER2+ subgroup and lack of targeted treatment in the included group, further confirmation in larger cohorts of HER2+ and TNBC tumours is required alongside further functional assessment.

In conclusion, this study revealed and confirmed that elevated CD133 expression is associated with poor prognostic characteristics and poor survival outcome. Elevated expression of CD133 appears to play a role in the proliferation and progression of the aggressive HER2+ subtype of BC, and is therefore a potential therapeutic target [56]. The combination of CSC with CD133 expression may serve as a screening tool to monitor recurrence and predict prognosis which warrant further functional studies.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

Research involving human participants This study was approved by the Nottingham Research Ethics Committee 2 (Reference title: Development of a molecular genetic classification of breast cancer). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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