Breast Cancer Res Treat (2011) 127:639–648
DOI 10.1007/s10549-010-1019-4

PRECLINICAL STUDY

Microcephalin is a new novel prognostic indicator in breast cancer associated with BRCA1 inactivation

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Received: 21 June 2010 / Accepted: 23 June 2010 / Published online: 15 July 2010

Abstract The authors have investigated the expression of the microcephalin (MCPH1) protein to evaluate its prognostic importance in breast cancer. Microcephalin is a damage response protein involved in the regulation of BRCA1 and BRCA2. *BRCA1* mutations are often associated with basal-like breast cancer, which are also often negative for oestrogen receptor (ER), progesterone receptor (PR) and HER2. MCPH1 immunohistochemistry was performed on 319 breast cancers prepared as tissue microarray and correlated with pathology, survival, ER, PR, HER2, EGFR, CK5/6, CK14 and BRCA1 expression. After performing continuous data analysis, mean microcephalin expression decreased with increasing grade ($P < 0.006$). Mean microcephalin expression was lower in ER/PR negative ($P < 0.001$) and triple negative cancers ($P < 0.004$). Conversely, an association with HER2-positive cancers was also identified ($P < 0.034$). Reduced microcephalin also correlated with reduced nuclear BRCA1 staining ($P < 0.001$). No association was identified with basal markers. After dichotomising the data into low and high microcephalin expression, reduced expression was identified in 29% (93/319) of breast cancers. An association with low expression was identified in invasive ductal carcinomas with breast cancer-specific survival (BCSS) ($P = 0.052$). Multivariate analysis of ductal carcinomas showed that microcephalin, together with lymph node involvement and tumour size were independent predictors of BCSS ($P = 0.037$). Microcephalin expression is reduced in 29% of breast cancers, particularly in higher grade tumours and BRCA1-negative cases. Microcephalin is an independent predictor of BCSS in invasive ductal breast cancer patients and may prove to be a useful biomarker for the identification of aggressive breast cancers.

Keywords Microcephalin · Breast cancer · Immunohistochemistry · HER2 · Triple negative · BRCA1

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Introduction

Previously, we identified mutations in *MCPH1* a DNA damage response gene, as a cause of primary microcephaly [1]. The MCPH1 protein called microcephalin is also known as BRIT1 (BRCT-repeat inhibitor of hTERT expression) which was initially identified as a transcriptional repressor of human telomerase reverse transcriptase [2]. Microcephalin contains three BRCA1 carboxyl-terminal (BRCT) motifs which were originally identified in the *BRCA1* gene and have been identified in other proteins involved in DNA repair and cell cycle checkpoints [3]. The DNA damage response is complex, and MCPH1 appears to have a number of important roles in this process. In

response to ionising radiation and ultraviolet light, MCPH1 co-localises to DNA damage response foci with other DNA repair proteins such as γH2AX [4]. MCPH1 knockdown reduces the formation of foci by the DNA repair proteins MDC1, 53BP1 and ATM [5]. MCPH1 interacts directly with BRCA2 and regulates the amount of both BRCA2 and Rad51 at DNA repair sites [6]. SiRNA knockdown of MCPH1 expression decreases BRCA1 and CHK1 expression and impairs the ionising radiation-induced S and G2/M checkpoints [7, 8]. MCPH1 plays a role in G2-M checkpoint control by regulating CHK1 localisation [9]. These studies strongly implicated MCPH1 as an early mediator in the DNA damage response, regulating the recruitment of repair proteins to the site of damage and triggering both the ATM/ATR damage response signalling cascades. Recently, microcephalin has been shown to interact with the transcription factor E2F1, leading to alterations in the regulation of CHK1, BRCA1, RAD51, p73 and caspases [10].

The function of microcephalin in DNA repair and checkpoint control makes it a potential tumour suppressor gene [5, 11]. Deletions at the MCPH1 loci (8p23) are associated with tumour development and poor prognosis in a wide range of cancers including breast cancer [12]. Previously, comparative genomic hybridisation (CGH) studies have identified decreased MCPH1 DNA copy number in 72% (39/54) of breast cancer cell lines, and in 40% (35/87) of ovarian cancers. Decreased MCPH1 mRNA levels were also identified in 63% (19/30) of ovarian cancers. These findings support the hypothesis that MCPH1 is a tumour suppressor gene [5].

Breast cancer is a heterogeneous disease both clinically and genetically. Recently, breast cancer has been classified into molecular subgroups based on their gene expression profiles, which include luminal A and B, HER2-amplified tumours, basal-like tumours and the normal breast-like tumours [13]. These subgroups could also be identified using immunohistochemistry for hormone receptor, HER2 and basal markers [14, 15]. Interestingly, dysfunction of the BRCA1 pathway is frequently observed in basal-like breast cancers [16]. At least, three quarters of breast cancers arising in germ line BRCA1 mutation carriers have a basal-like phenotype by immunohistochemistry [17] or gene expression microarray [13]. Basal-like breast cancers express basal/myoepithelial markers such as cytokeratin 5/6, 14, 17 or vimentin and EGFR (HER1) [15, 18]. This subtype of breast cancer has an aggressive phenotype, poor prognosis and lack expression of the receptors oestrogen alpha (ER), progesterone (PR) and HER2 [15, 18], often referred to as triple negative. Potentially, owing to the function of microcephalin in DNA repair, particularly the BRCA1 pathway, reduced microcephalin expression may be associated with the basal-like phenotype.

In this study, we have investigated microcephalin expression in a large well-characterised series of breast cancer samples with long-term follow up to determine its clinical and biological relevance. The association of microcephalin expression with the biomarkers ER, PR, HER2, EGFR, CK5/6, CK14 and BRCA1 has also been investigated.

Materials and methods

Patients

This study involves two independent cohorts of breast cancer cases. The first cohort containing 65 cases from Leeds Teaching Hospitals Histopathology archives was designated as the training set, and the second cohort containing 319 cases from Nottingham University Hospitals NHS Trust Histopathology archives was used as the validation set. The Leeds cases were unselected from patients presenting between 1987 and 2004. The Nottingham cases were unselected from patients presenting between 1988 and 1998. Clinical history and tumour characteristics (age, tumour type, size, histological grade, lymph node status and NPI) were available for both series. In both sample cohorts, DFS was defined as the interval (months) from primary surgical treatment to the first loco regional or distant recurrence. BCSS was taken as time (months) from primary surgical treatment to time of death due to breast cancer. Patients received Tamoxifen or classical cyclophosphamide, methotrexate and 5-fluorouracil chemotherapy depending on their ER status. The Nottingham series is well characterised with data available for other biomarkers involved in breast cancer including ER, PR, HER2, EGFR, BRCA1 and basal cytokeratins (CK) 5/6 and 14 [14, 19]. Ethical approval for this study has been obtained from the Local Research Ethics Committee of the Leeds Teaching Hospitals NHS Trust, and from the Nottingham Research Ethics Committee 2. The reporting recommendations for tumour marker prognostic studies (REMARK) criteria, as recommended by Mc Shane et al. [20] have been followed throughout this study.

Immunohistochemistry

In order to optimise the MCPH1 staining protocol, full sections of paraffin-embedded tissue from both normal and breast tumour samples were stained using a range of primary antibody dilutions, incubation times and antigen retrieval methods. MCPH1 was stained on full sections of patient's samples in the training set, whilst in the validation set, TMAs were constructed as previously described [14]. Immunohistochemical analysis of microcephalin expression was performed with the rabbit anti-microcephalin

antibody (BL1610, Bethyl Laboratories) at a 1:50 dilution on full sections and at 1:100 on the TMA. In order to avoid reduction in immunoreactivity of tissue sections over time, the sections were cut just before staining.

TMA sections of four micrometer were deparaffinised in graded alcohols. Endogenous peroxide activity was inhibited by immersing the sections in 0.5% H_2O_2. Antigen retrieval was carried out by pressure cooking slides for 2 min at full pressure in 1% Antigen Unmasking Solution (Vector Laboratories Ltd, Peterborough, UK). A casein incubation (1/2 dilution) was also carried out to reduce non-specific staining (Vector Laboratories). Sections were incubated with the antibody for 2 h at room temperature. Bound antibody was detected with EnVision™ polymer (DAKO, Ely, Cambridgeshire, UK), using diaminobenzine as the substrate (Sigma, Poole, Dorset, UK). Sections were counterstained with Mayer's haematoxylin (VWR International Ltd, Poole, England). Negative controls, without primary antibody and positive controls of normal breast tissue, were included in each batch of immunohistochemistry.

Immunohistochemical evaluation

The TMA sections were scored using high resolution digital images (NanoZoomer, Hamamatsu Photonics, Welwyn Garden City, UK), at 20× magnification, using a web-based interface (Distiller, Slidepath Ltd, Dublin, Ireland). Nuclear and cytoplasmic staining was scored as a percentage of the positive cells in relation to the total number of tumour cells present. All the samples were scored by one observer (SMB), and a representative sample was counter-scored by a specialist consultant breast histopathologist (AMS) to ensure reproducibility. The immunoreactivity, scoring and categorisation of ER, PR, HER2, EGFR, CK5/6, CK14, BRCA1 and triple negative phenotype were defined in this series as previously described [14, 19].

Statistical analysis

The percentage of cells with nuclear microcephalin staining was evaluated as a continuous variable to avoid the loss of information that results from categorisation of continuous data [21, 22]. However, for completeness, the percentage of nuclear microcephalin staining was also dichotomised by sequentially testing every cut-off value versus BCSS using the Kaplan–Meier curves. The cut-off point which showed the highest significance between patient groups with regard to BCSS was used for subsequent analyses. Statistical analysis was performed using SPSS version 16.0, (SPSS Inc, Chicago, IL, USA). Pearson correlation was used to correlate microcephalin staining as a percentage of the positive cells versus tumour size, age of patient, number of positive nodes, vascular invasion, BCSS duration and DFS duration as continuous variables. The Chi-square test/Fisher exact test or Mann–Whitney *U*-test (non-parametric) was used for comparisons amongst groups/categorical data as appropriate. Survival curves were generated using the Kaplan–Meier method, and differences amongst groups were analysed by the log-rank test and confirmed by applying the Cox regression model. Previously, published cut-off values were used for the established prognostic factors in this series of patients [14, 19]. For systematic modelling, a forward stepwise multivariable Cox regression model was used, testing the independent prognostic relevance of microcephalin immunoreactivity. All the statistical tests were two-sided, and a $P \leq 0.05$ value was considered statistically significant.

Results

Patient's characteristics

This study included two groups of patients after excluding core loss and unscoreable cores. The first group had 65 patients and is considered as a training set, whilst the second group had 319 patients and is considered as a validation set. Detailed patients' characteristics of the validation set are summarised in Table 1.

Since this is the first large immunohistochemistry study on microcephalin expression in breast cancer, no prior cut-off point was available; therefore, two types of analysis were performed. Initially, the percentage of cells with nuclear microcephalin staining was treated as continuous data. In addition, we dichotomised the percentage of nuclear microcephalin staining. A cut-off point of 35% positively stained cells showed the most significant difference between the two groups of patients (low and high microcephalin) in BCSS in the validation study. There was insufficient follow-up information to meet the statistical analysis requirements in the training set. However, in the validation set, during a median follow-up period of 124 months (range 5–229 months), death occurred in 101/293 patients (34.5%), and metastasis or/and recurrence occurred in 133/293 patients (45%).

Microcephalin expression in normal and tumour breast tissues

Initially, the expression of microcephalin was evaluated in whole breast cancer sections ($n = 65$). Strong nuclear staining was identified in normal breast samples (Fig. 1a). In the tumour samples, microcephalin was expressed in both the nucleus and cytoplasm of tumour cells (Fig. 1b). In many cases, strong nucleolar staining was also detected in the nuclei (Fig. 1c). Interestingly, in a small number of

Table 1 Patients' characteristics

Variables	Validation set $n = 319$ (%)
Age	
Mean	53
Range	28–70
Age distribution	
<50	117 (36.7)
>50	202 (63.3)
Tumour size (mm)	
Mean	20
Range	2.7–100
Size distribution (mm)	
<20	158 (49.7)
>20	160 (50.3)
Tumour grade	
G1	58 (18.2)
G2	97 (30.5)
G3	163 (51.3)
Lymph node status	
1	188 (59.1)
2	97 (30.5)
3	33 (10.4)
Vascular invasion (VI)	
No	174 (54.9)
Probable	27 (8.5)
Definite	116 (36.6)
Nottingham prognostic index (NPI)	
Good	75 (23.6)
Moderate	193 (60.7)
Poor	50 (15.7)
Histological tumour type	
Ductal/NST	191 (61)
Lobular	30 (9.6)
Tubular and tubular mixed	62 (19.9)
Medullary	7 (2.2)
Other special types[a]	4 (1.3)
Mixed[b]	19 (5.5)
Distant metastases	
No	207 (65.1)
Positive	111 (34.9)
Recurrence	
No	164 (52.3)
Yes	150 (47.7)
Endocrine therapy	
Not given	187 (59)
Given	120 (38)
Unknown	12 (3)
Chemotherapy	
Not given	239 (76)
Given	68 (21)
Unknown	12 (3)

[a] Includes mucoid and invasive papillary carcinoma

[b] Includes ductal/NST mixed with lobular or special types

tumour samples (3/65), only cytoplasmic microcephalin staining was detected (Fig. 1d).

Dichotomous analysis in the training set identified low microcephalin expression in 22/65 (34%) samples. Figure 1e shows a grade 3 invasive ductal carcinoma showing loss of microcephalin expression. In the validation set, 93/319 (29%) of cases showed low microcephalin expression. Low and high microcephalin expression in TMA samples with IDC are shown in Fig. 1 (f, g). The adjacent normal epithelium which was used as an internal control showed high microcephalin nuclear staining in the majority of cases.

Relation of microcephalin expression to histological parameters

In each patient sample, the level of microcephalin expression was correlated with the severity of the disease. Continuous data analysis of the validation set identified a highly significant association between low expression of microcephalin and high tumour grade ($P < 0.003$). Mean microcephalin expression was 61% in grade 2 tumours compared to 50% in the high grade 3 tumours. Microcephalin expression did not differ significantly in the distribution of other clinicopathological variables. Similar results were obtained after dichotomous analysis into low and high microcephalin expression; however, the statistical significance was reduced (Table 2).

Association of microcephalin expression with biomarkers

Continuous data analysis of microcephalin nuclear expression identified significant correlations with the ER, PR and HER2. Microcephalin expression was significantly associated with the negative expression of ER and PR ($P < 0.001$). Mean microcephalin expression was 45.2% in ER-negative cases compared to 57% in positive cases. Similarly, mean microcephalin expression was 46.5% in PR-negative cases compared to 59% in positive cases. In the triple negative phenotype (ER, PR and HER2), mean microcephalin expression was 55.8% compared to 43.7% ($P < 0.004$). Conversely, mean microcephalin expression was significantly lower (43.8) in HER2-positive tumours than HER2-negative ones (55.4%) ($P < 0.034$).

Continuous data analysis of microcephalin nuclear expression identified a significant difference with absent or reduced nuclear BRCA1 staining ($P < 0.001$). Mean microcephalin expression was 30.6% in BRCA1-negative cases compared to 52.7% in positive cases with reduced BRCA staining, and 63.3% with strong nuclear BRCA1 staining. No significant correlation was identified between

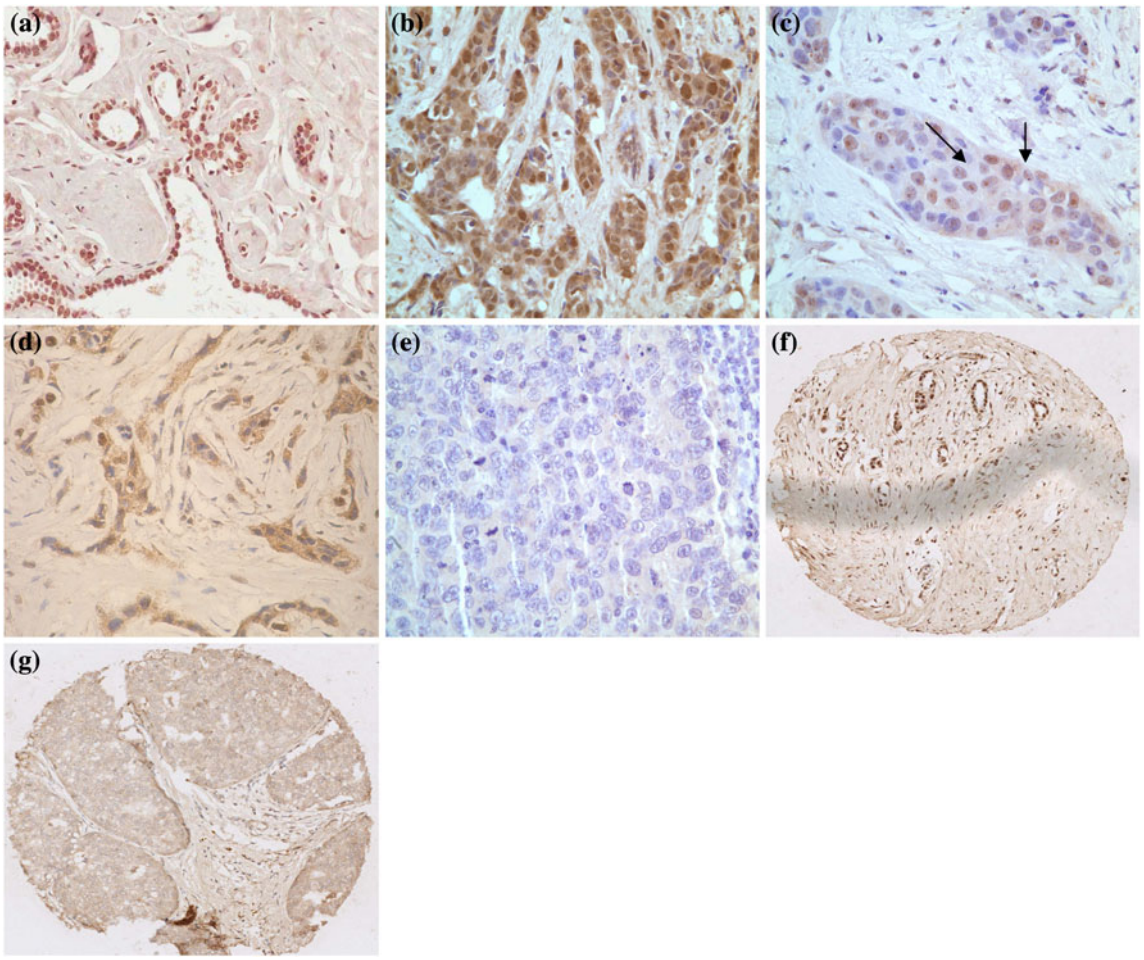

Fig. 1 Immunohistochemical analysis of microcephalin expression in normal and malignant breast tissue samples. **a** Normal mammary ducts showing strong nuclear expression of microcephalin (×20). **b** Grade 1 invasive ductal carcinoma of no special type (NST) showing nuclear and cytoplasmic microcephalin staining (×20). **c** Invasive ductal carcinoma NST showing nucleolar expression of microcephalin (*arrows*) (×40). **d** Grade 3 invasive ductal carcinoma NST with no nuclear microcephalin expression. Cytoplasmic staining is, however, present (×40). **e** Grade 3 invasive ductal carcinoma NST, with no microcephalin expression (×40). **f** Grade 1 invasive ductal carcinoma TMA showing high-level microcephalin expression (×10). **g** Grade 3 invasive ductal carcinoma TMA showing no nuclear microcephalin expression and low-level cytoplasmic staining (×10)

microcephalin expression and cytoplasmic BRCA1 staining or the basal markers CK14, CK5/6 and EGFR. Similar results were obtained when the data were dichotomised into low and high microcephalin expression; however, the statistical significance was reduced. The correlation of microcephalin expression and other biomarkers is shown in Table 3.

Univariate and multivariate analyses

In the validation set, continuous data analysis of microcephalin expression identified no correlation with BCSS. However, after dichotomisation, a weak association with reduced microcephalin expression and shorter BCSS was identified. In patients with low microcephalin expression, the mean BCSS was 146.6 months (95% CI: 128–164) compared to 154.8 months (95% CI: 143.9–165.7) in patients with high microcephalin expression ($P = 0.103$) (Fig. 2a). This reached statistical significance in invasive ductal carcinomas alone (HR = 0.6, 95% CI: 0.4–1, $P = 0.05$) (Table 4). Mean BCSS of patients with low expression of microcephalin was 128.7 months compared to 146.7 months in patients with high microcephalin expression (Fig. 2b). There was no significant association between microcephalin expression and DFS (Fig. 3).

A multivariate Cox regression model was carried out to examine the independent prognostic impact of microcephalin expression on BCSS and DFS in ductal carcinomas in relation to other established clinicopathological and tumour-biological factors such as tumour grade, size, lymph node status, NPI, vascular invasion, adjuvant hormonal and chemotherapy. In a systematic model,

Table 2 Correlation of microcephalin expression with clinicopathological data in the validation set

Parameter	Microcephalin expression				
	Continuous		Categorised[a]		
	Mean (%)	*P* value	High *n* (%)	Low *n* (%)	*P* value
Age					
<50	56	0.631	84 (37)	33 (35)	0.799
>50	53		141 (63)	61 (65)	
Tumour size (mm)					
<20	56.7	0.197	116 (52)	42 (45)	0.248
>20	52.2		108 (48)	52 (55)	
Tumour grade					
G1	54.8	G1 vs. G2 0.282	40 (18)	18 (19)	0.311
G2	61.1	G2 vs. G3 **0.003**	74 (33)	23 (24)	
G3	50.4	G1\2 vs. G3 **0.006**	110 (49)	53 (56)	
Lymph node stage					
1	53.2	1 vs. 2 0.645	126 (56)	61 (65)	0.305
2	55.8	1 vs. 3 0.754	72 (32)	26 (28)	
3	57.9	2 vs. 3 0.989	26 (12)	7 (7)	
Vascular invasion (VI)					
No	54.3	N vs. P 0.547	120 (54)	54 (57.5)	0.820
Probable	51	N vs. D 0.835	19 (8.5)	8 (8.5)	
Definite	55.6	P vs. D 0.431	84 (37.5)	32 (34)	
Nottingham prognostic index					
Good	56.2	G vs. M 0.696	52 (23)	22 (24)	0.737
Moderate	55.1	G vs. P 0.145	139 (63)	55 (59)	
Poor	49.4	M vs. P 0.231	33 (14)	17 (18)	
Histological tumour type					
Ductal/NST	51.8	[d]D vs L **0.019**	130 (57)	61(72)	0.413
Lobular	63.6	L vs T 0.090	23 (10)	7 (8)	
Tubular and tubular mixed	54.6	T vs D 0.200	51 (22)	11 (13)	
Medullary	44.7		7 (3)	0 (0)	
Other special types[b]	53.3		3 (1)	1 (1)	
Mixed[c]	46.6		14 (6)	5 (6)	
Distant metastases					
No	53.6	0.627	144 (64)	63 (67)	0.700
Positive	56.3		80 (36)	31 (23)	
Recurrence					
No	52.4	0.266	112 (50.5)	52 (57)	0.385
Yes	56.9		110 (49.5)	40 (43)	

[a] A 35% cut off was used to categorise microcephalin expression into low and high groups

[b] Includes mucoid and invasive papillary carcinoma

[c] Includes ductal/NST mixed with lobular or special types

[d] Statistical analysis was only performed for most common subtypes

$P \leq 0.05$ is significant and are shown in bold

microcephalin, together with lymph node involvement and tumour size, was independent predictors of BCSS in invasive ductal carcinomas alone (HR = 0.6, 95% CI: 0.4–1, $P = 0.037$) (Table 4).

In the small cohort of patients treated with chemotherapy (59), the mean BCSS of patients with tumours expressing microcephalin was 145 months compared to 112 months in patients with tumours having reduced

Table 3 Correlation of microcephalin expression with other biomarkers

Parameter	Microcephalin expression				
	Continuous		Categorised[a]		
	Mean (%)	*P* value	High *n* (%)	Low *n* (%)	*P* value
ER					
Negative	45.2	**0.001**	55 (27)	33 (35)	0.117
Positive	57		152 (73)	60 (65)	
PR					
Negative	46.5	**0.001**	80 (39)	49 (54)	**0.018**
Positive	59		125 (61)	42 (46)	
HER2					
Negative	55.4	**0.034**	184 (90)	72 (82)	0.199
Positive	43.8		22 (10)	16 (18)	
Triple negative					
Negative	55.8	**0.004**	173 (84)	70 (76)	0.126
Positive	43.7		33 (16)	22 (24)	
EGFR					
Negative	54.65	0.863	149 (79)	61(77)	0.769
Positive	53.37		40 (21)	18 (23)	
CK5/6					
Negative	54.87	0.521	185 (87)	75(82)	0.314
Positive	51.18		28 (13)	16 (18)	
CK14					
Negative	54.13	0.535	178 (86)	78 (85)	0.858
Positive	50.9		30 (14)	14 (15)	
BRCA1 (nuclear)					
Negative	30.6	**0.001** N vs. R	15 (8)	23 (29)	**<0.001**
Reduced	52.7	**0.010** R vs. S	72 (39)	31 (39)	
Strong	63.3	**<0.001** N vs. S	101 (54)	25 (32)	
BRCA1 (cytoplasmic)					
Negative	57.6	0.413	124 (85)	57 (47)	0.323
Positive	53.1		22 (15)	64 (53)	

[a] A 35% cut off was used to categorise microcephalin expression into low and high groups

$P \leq 0.05$ is significant and are shown in bold

microcephalin expression. This difference, however, was not statistically significant. No significant correlation was identified between microcephalin expression and response to hormone therapy.

Discussion

In this study, we have undertaken the first large-scale investigation of the expression of the MCPH1 protein microcephalin in breast cancer samples. In normal breast duct epithelial cells, nuclear and nucleolar microcephalin stainings were observed. The nuclear localisation is consistent with the role of microcephalin in DNA repair, checkpoint and transcription regulation. Nucleolus localisation is often seen in DNA repair proteins such as BRCA1, which are stored in the nucleolus before relocalisation to DNA damage foci when required [23]. In the tumour cells, nuclear (including nucleolar) and/or cytoplasmic microcephalin staining was identified. Interestingly, in a small number of high grade tumour samples, only cytoplasmic microcephalin staining was detected.

Initially, we recorded low microcephalin expression in 34% (22/65) of the training set. Our larger validation set also showed a reduction in microcephalin expression of 29% (93/319). Our results agree with the other studies which report reduced MCPH1 DNA copy number in 72% (39/54) of breast cancer cell lines and also reported reduced microcephalin staining in 7/10 breast cancer samples [5, 24].

Our results revealed that low microcephalin expression was significantly associated with high tumour grade in our validation set. In the validation set, reduction of microcephalin expression was weakly associated with shorter BCSS. This association with shorter BCSS was even more obvious amongst invasive ductal carcinomas, which is the main histological subtype of breast cancer with a highly variable prognosis, and, therefore, more accurate prognostic factors are needed especially for this histological breast cancer subtype. The multivariate Cox regression model showed that microcephalin levels and lymph node involvement and tumour size are independent predictors of BCSS in invasive ductal breast cancers. This finding is similar to a recent breast cancer study which identified an association between decreased MCPH1 transcript levels and reduced time to metastasis [5].

In this study, low microcephalin immunohistochemical expression correlated with the triple negative phenotype, and conversely positive HER2 status. Morphologically, the triple negative and HER2-positive cancers tend to be aggressive high grade cancers. Since reduced microcephalin expression is significantly associated with increasing tumour grade it is likely that microcephalin expression may be a marker of poor differentiation rather than that of a particular subtype.

Interestingly, reduced microcephalin expression also correlated with no or reduced nuclear BRCA1 expression. This finding is in agreement with previous DNA repair studies which have reported that MCPH1 siRNA knockdown causes reduced BRCA1 expression in a range of cell lines [7, 8, 10, 11]. To our knowledge, this is the first study to confirm the association between reduced microcephalin and BRCA1 expression in breast tumour samples. A potential explanation for this association is that microcephalin is a positive regulator of *BRCA1* due to its interaction with the transcription factor E2F1 on the promoter

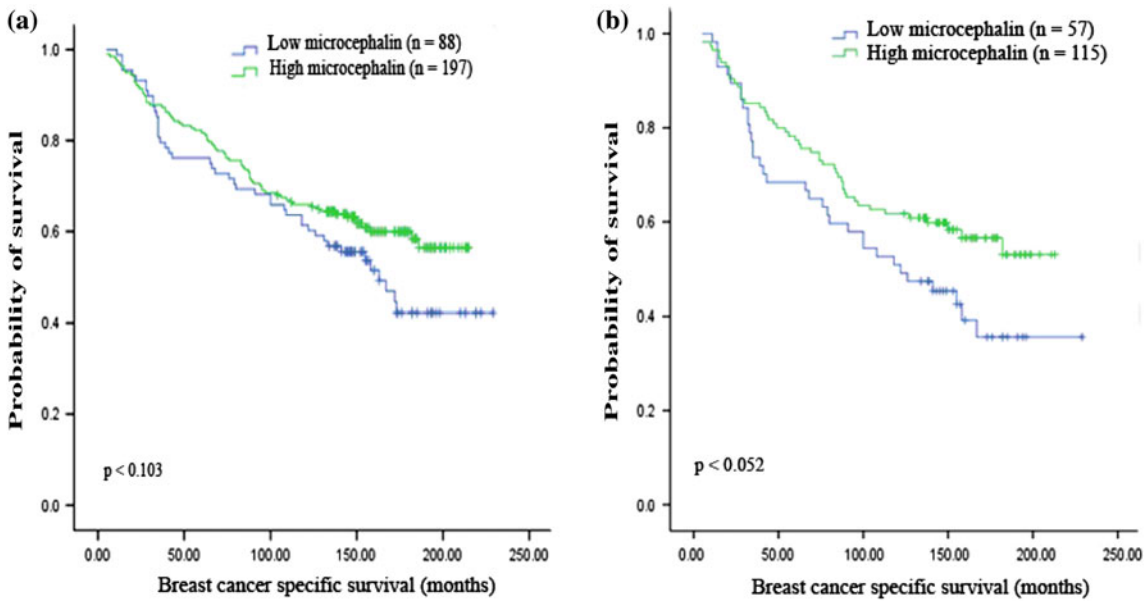

Fig. 2 Comparison of breast cancer-specific survival based on microcephalin status: **a** whole patient series, and **b** ductal breast cancer patients only

Table 4 Univariate and multivariate analyses of breast cancer-specific survival in the validation set: (a) the whole patient series, and (b) ductal breast cancer patients only

Variable	Univariate (*P* value)	HR (95% CI)	Multivariate (*P* value)	HR (95% CI)
(a) Whole patient series				
Grade	0.009	1.4 (1.1–1.8)	0.004	1.7 (1.2–2.6)
Lymph node status	<0.001	1.8 (1.4–2.3)	<0.001	2.3 (1.5–3.5)
Size	0.001	2 (1.4–2.9)	0.001	1.6 (1–2.2)
NPI	<0.001	1.9 (1.45–2.6)	0.335	0.7 (0.4–1.3)
Vascular invasion	<0.001	1.3 (1–1.6)	0.872	1 (0.7–1.3)
Chemotherapy	0.771	1 (0.7–1.7)	0.007	0.4 (0.2–0.8)
Endocrine therapy	0.248	1.2 (0.9–1.8)	0.131	0.7 (0.4–1)
Microcephalin expression[a]	0.103	0.74 (0.5–1.1)	0.064	0.7 (0.5–1)
(b) Ductal breast cancer patients only				
Grade	0.389	1.1 (0.7–1.6)	0.381	1.3 (0.7–2.3)
Lymph node status	**<0.001**	2.2 (1.6–3)	<0.001	3.3 (2–5.3)
Size	**0.004**	1.9 (1.2–3)	0.005	1.5 (1.1–2.1)
NPI	**<0.001**	2 (1.3–3)	0.337	0.7 (0.33–1.5)
Vascular invasion	0.582	1 (0.8–1.5)	0.125	0.75 (0.5–1.)
Chemotherapy	0.712	0.9 (0.55–1.5)	0.022	0.4 (0.2–0.9)
Endocrine therapy	0.438	1.2 (0.8–1.9)	0.339	0.75 (0.4–1.3)
Microcephalin expression[a]	0.052	0.6 (0.4–1)	0.037	0.6 (0.4–1)

Multivariate analysis: (a) total $n = 275$, deceased = 116, censored = 159, missing = 48, and (b) total $n = 169$, deceased = 80, censored = 89, missing = 22

[a] A 35% cut off was used to dichotomise microcephalin expression into low and high groups

$P \leq 0.05$ is significant and are shown in bold

of the *BRCA1* gene increasing transcription [10]; consequently, reduced microcephalin expression would result in reduced *BRCA1* expression. Many sporadic breast cancers may show decreased BRCA1 expression and an aggressive BRCA1-like phenotype; however, this can only be partially accounted for by methylation, mutations and deletions

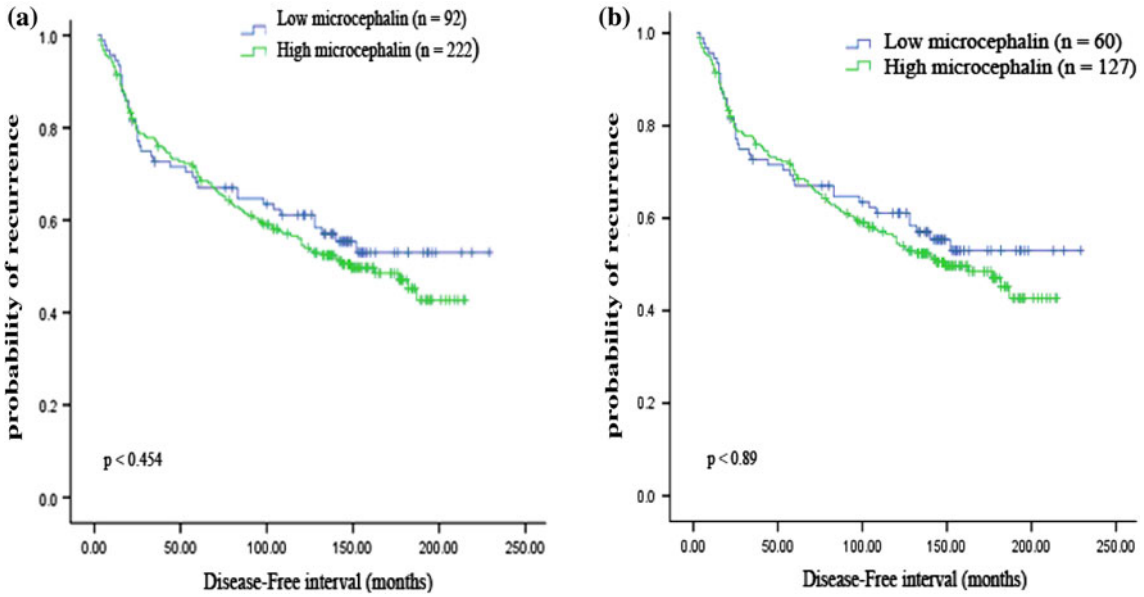

Fig. 3 Comparison of disease-free survival based on microcephalin status: **a** whole patient series, and **b** ductal breast cancer patients only

[16, 25], reduced expression of positive regulators like microcephalin could be another mechanism.

Recently, the use of Parp inhibitors has proved to be very effective in the treatment of patients with breast and ovarian cancers containing *BRCA1/2* mutations [26]. The Parp-1 enzyme plays an important role in base excision DNA repair, when this function is inhibited in cells with compromised homologous recombination DNA repair, such as BRCA1/2-deficient cells; the cells are more sensitive to the increased DNA damage and apoptosis occurs [26]. Potentially, Parp inhibitors may prove to be useful in treating microcephalin deficient cancers due to the association between loss of microcephalin expression and decreased BRCA1 expression, and the requirement for microcephalin in BRCA2/RAD51 mediated repair [6].

Reduced expression of microcephalin was associated with aggressive high grade tumours and poor patient outcome particularly in invasive ductal breast cancers. Potentially, reduced expression of the DNA repair protein microcephalin could cause resistance to chemotherapy. Unfortunately, the number of patients used in this study was not large enough to determine if microcephalin had an effect on chemotherapy response. However, the 33 month's lower survival in patients with low microcephalin expression identified in this study indicates that further studies are warranted. In addition to regulating BRCA1 expression through binding E2F1, microcephalin also alters the expression of p73, caspase 3 and 7, CHK1 and RAD51 which may also influence response to chemotherapy [10].

Recently, BRIT1/MCPH1 knockout mice models have been developed. In one model, deregulated mitosis and premature chromosome condensation was identified and, in the other, deficient DNA repair [27, 28]. Whilst to date, no signs of cancer development have been detected in either model, Liang et al. reported that when the DNA repair-deficient BRIT1/MCPH1 mice were crossed with p53 knockout mice, this resulted in a significant increase in susceptibility to cancer. The authors also reported that low dosages of irradiation induced breast tumours in mice with conditional knockout of BRIT1 in the mammary glands, but not in control littermates [28]. Our results identifying reduced microcephalin expression in HER2-amplified and BRCA1-negative breast cancers suggest that it would be very interesting to cross the BRIT1/MCPH1-deficient mice with amplified HER2 and BRCA1 mice to determine the influence of microcephalin expression on tumour development.

Further, large confirmatory studies are warranted to determine the potential use of microcephalin expression assessment in clinical practice in breast and other cancer types. In summary, reduction of microcephalin expression was identified in almost one third of breast carcinoma cases particularly in higher grade tumours and BRCA1-negative cases. Although reduced microcephalin expression was identified in triple negative and HER2-positive cancers, this association may simply reflect the aggressive nature of these subtypes. Multivariate analysis which identified microcephalin as an independent prognostic factor for BCSS provides clinical evidence to support the idea that microcephalin is a tumour suppressor gene, which may prove to be a new prognostic biomarker in aggressive breast cancers.

Acknowledgements This study was supported by grants from Yorkshire Cancer Research grant number L317 (SMB & VS), and the Breast Cancer Campaign 2007NovPR53 (SMB & VS). MK is supported by a scholarship from the Egyptian government. RA is supported by a scholarship from the Saudi Arabian government.

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