

## CD44 is overexpressed in basal-like breast cancers but is not a driver of 11p13 amplification

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**Abstract** Overexpression and alternative splicing of CD44 have been implicated in tumour progression. Here we describe the identification of a high level amplification of human 11p13, encompassing the *CD44* gene, in primary breast cancers and cell lines and test whether *CD44* acts as the driver of this amplicon. aCGH analysis revealed 11p13 amplification in 3% (3/100) of primary breast carcinomas and in two cell lines. The minimal region of amplification was 34.38–37.62 Mb. Amplification was confirmed by dual-colour FISH in these cell lines and further validated by CISH in an independent tumour cohort. CD44 expression in primary breast cancers was significantly associated with features of basal-like breast cancer. Detection of CD44 expression in breast cancer cell lines confirmed

moderate to high expression in basal-like cell lines and minimal expression in luminal cell lines. In both, primary breast cancers and cell lines, 11p13 amplification was associated with high levels of CD44 mRNA expression. CD44 alternative splicing was detected in four of nine cell lines and in tumour samples, irrespective of the amplification status. RNAi mediated knock down of CD44 failed to reveal an increased dependence on CD44 expression for proliferation or survival in amplified cell lines. Given that expression of CD44 is not an absolute requirement for the survival of cells harbouring *CD44* gene amplification, CD44 is unlikely to be a driver of the 11p13 amplicon.

**Keywords** CD44 · Amplification · Breast cancer · Basal-like · 11p13

Pamela Klingbeil and Rachael Natrajan have contributed equally to this work. RN and RV performed aCGH and data analysis and RV performed FISH and CISH. CM and JSR-F interpreted the immunohistochemical and in situ hybridisation experiments. JP and HB provided the breast cancer samples for aCGH. PK and GE performed all the cell based assays. CMI and JSR-F designed the experiments. PK, RN, JSR-F and CMI wrote the manuscript.

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### Abbreviations

aCGH	Array chromosomal genomic hybridisation
CISH	Chromogenic in situ hybridisation
Ck	Cytokeratin
ER	Oestrogen receptor
EGFR	Epidermal growth factor receptor
FISH	Fluorescent in situ hybridisation
PR	Progesterone receptor
qRT-PCR	Quantitative real-time polymerase chain reaction

### Introduction

The human *CD44* gene locus maps to 11p13 and contains 20 coding exons [1, 2]. Excessive alternative splicing and posttranslational modifications give rise to a family of transmembrane glycoproteins with diverse structural and functional properties. The standard CD44 isoform (CD44s)

contains none of the alternatively spliced exons (variant exons 1–10) and is expressed by nearly all cell types. In contrast, the alternatively spliced, variant isoforms (CD44v) show a highly restricted expression pattern during embryonic development, in pathological backgrounds or in a cell type specific manner, for example, epithelial cells mainly express CD44v8-v10.

CD44 has been implicated in many steps of tumour progression: proliferation, migration, invasion and apoptosis resistance, and at least in part these events are mediated by CD44 binding to its major ligand, the extracellular matrix glycosaminoglycan, hyaluronan [1, 2]. Of particular interest are the numerous reports connecting expression of variant CD44 isoforms and development of metastasis. These in vitro and in vivo studies have led to a number of investigators examining whether there is a clinical correlation linking CD44 expression with patient prognosis and response. These different studies have all reported that a proportion of breast cancers showed elevated expression of CD44, although there are conflicting reports as to whether this expression correlates with particular subtypes of breast cancer and the pattern of variant CD44 isoform expression [3–7]. With respect to patient outcome, again a consistent trend has failed to emerge. For example, it has been reported that there is a significant correlation between CD44s expression and increased disease-free survival in node-negative patients [8] and that expression of CD44v7-8 correlates with decreased overall survival [6]. Similarly it has been reported both that CD44v6 is and is not a poor prognostic marker [6, 9]. The difficulty in these studies is the complexity of the CD44 variant isoform expression patterns and the lack of reagents to accurately monitor expression of the different splice variants. Together with the lack of evidence that CD44 is an independent prognostic marker, further progress in analysing CD44 expression patterns in breast cancer has been limited.

Recent interest in CD44 has stemmed from reports that high levels of CD44 expression in combination with low level expression of CD24 can be used to prospectively identify a population of breast cancer cells enriched in stem cell-like properties [10]. Similarly, expression of CD44+/CD133+/ $\alpha$ 2 $\beta$ 1+ or CD44+/EpCAM+ have been used to identify a colorectal cancer stem cell population [11, 12]. Subsequently a number of studies have demonstrated that the CD44<sup>high</sup>/CD24<sup>low/negative</sup> phenotype in cells cultured from primary cancers or expressed in subpopulations of established breast cancer cell lines, identified a population with stem/progenitor-like properties [13–15]. Of particular interest has been the proposal that these CD44+ve subpopulations are mediators of metastasis and that *CD44* may act as a metastasis promoting gene.

Gene amplification is one of the underlying genetic mechanisms leading to overexpression of genes in cancer

cells when compared to their normal counterparts. Compared to extensive reported data on the function of CD44 in vitro and its expression in vivo, there have been no systematic study on *CD44* gene amplification and the potential implications thereof. Here we describe an amplification on 11p13 spanning the *CD44* locus in primary breast cancer samples as well as in established breast cancer cell lines and test a potential role for *CD44* as an amplicon driver. Amplification of 11p13 correlates with high expression levels of CD44 but had no clear influence on alternative splicing regulation of this gene. Cell lines harbouring the *CD44* amplification are not dependent on CD44 expression, arguing for another gene within the amplicon as amplicon driver.

## Materials and methods

### Cell lines

All cell lines were cultured in the presence of 100 U/ml penicillin, 10  $\mu$ g/ml streptomycin and 10% foetal bovine serum (FBS), with the exception of SUM149 cells, which were cultured in 5% FBS. Culture conditions were maintained at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere. MDA-MB-468, HS578T, MDA-MB-157, MDA-MB-231 and MCF7 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Paisley, UK). BT474 cells were cultured in the American Tissue Culture Collection (ATCC) Hybricare medium (46-X) supplemented with 1.5 g/l sodium bicarbonate. HCC1500 and HCC1954 cells were cultured in the RPMI-1640 medium (Invitrogen). SUM149 cells were cultured in DMEM/Ham's F12 medium (Invitrogen) supplemented with 5  $\mu$ g/ml recombinant human insulin (Sigma, Dorset, UK) and 1  $\mu$ g/ml hydrocortisone (Sigma). For immunofluorescent staining and Western blot analysis, the pan anti-human CD44 monoclonal antibody E1/2.8 [16] which recognises all CD44 splice variants was used.

### Sample collection and characterisation

A consecutive series of 104 grade III invasive ductal carcinomas of no special type (GIII-IDC-NST) cases was retrieved from the University Hospital La Paz, Madrid, Spain and the University of Munster, Germany. This project has been approved by the local Research Ethics Committees of the authors' institutions. Representative 4  $\mu$ m thick histological sections of each tumour were cut and stained with antibodies against oestrogen receptor (ER), HER2, epidermal growth factor receptor (EGFR) and cytokeratin (Ck) 5/6 as previously described [17, 18].

An independent series of 245 invasive breast carcinomas was arrayed in a tissue microarray (TMA) constructed with replicate 0.6 mm cores. All patients were diagnosed and managed at the Royal Marsden Hospital (RMH) between 1994 and 2000. Patients were selected on the basis of being eligible for therapeutic surgery, being followed up at the RMH, having representative histological blocks in the RMH pathology files, and receiving standard anthracycline-based adjuvant chemotherapy. Adjuvant endocrine therapy was prescribed for patients with ER positive tumours (tamoxifen alone in 96.4% of the patients for the available follow-up period). Complete follow-up was available for 244 patients, ranging from 0.5 to 125 months (median = 67 months, mean = 67 months). Tumours were graded according to a modified Bloom–Richardson scoring system [19] and size was categorised according to the TNM staging criteria [20]. The study was approved by the Royal Marsden Hospital Ethics Committee. 3 µm sections from the TMAs were mounted on polylysine-coated slides and immunohistochemistry was performed with antibodies raised against the following markers: ER, progesterone receptor (PR), HER2, EGFR, Ck 5/6, Ck 14, Ck 17, Cyclin D1, KI67, p53, topoisomerase II  $\alpha$ , caveolin 1 (CAV1), caveolin 2 (CAV2) and FOXA1 as reported elsewhere [21]. TMA sections were also subjected to chromogenic in situ hybridisation (CISH) with SpotLight probes for *CCND1*, *MYC*, *HER2*, *TOP2A*, and chromosome 8 centromere as previously reported [21–23].

#### Microarray CGH (aCGH)

Five consecutive 8 µm thick frozen sections of GIII-IDC-NST tumours were microdissected as previously described [24, 25] to obtain a percentage of tumour cells in the remaining tissue greater than 90%. The DNA concentration in tumours and cell lines was measured with Picogreen® according to the manufacturer's instructions (Invitrogen, Paisley, UK) and optimal DNA quality was objectively assessed using a previously described multiplex quality control PCR [26]. Microarray-based comparative genomic hybridisation (aCGH) was successfully performed in 100 consecutive fresh/frozen samples (four cases were excluded due to insufficient DNA quantity) and nine breast cancer cell lines using the 32 K BAC re-array collection (CHORI) tiling path aCGH platform [24]. This type of BAC array platform has been shown to be as robust as and to have comparable resolution with high density oligonucleotide arrays [27–29]. DNA labelling, hybridisations, washes, slide scanning and data acquisition were performed as previously described [24], and data analysed as described in detail elsewhere [18]. Thresholds were applied smoothed Log<sub>2</sub> ratios, which were converted into categorical data as previously described [18], with low level

gain defined as a smoothed Log<sub>2</sub> ratio of between 0.08 and 0.4, corresponding to approximately 3–4 copies of the locus, whilst gene amplification was defined as having a Log<sub>2</sub> ratio >0.4, corresponding to more than 5 copies [18, 24, 25]. A detailed analysis of the profiles of the IDC-NSTs is described elsewhere [18]. Tumours were classified into simplex, complex 'sawtooth' and complex 'firestorm' as previously described [18, 25, 30].

CD44 immunohistochemical, chromogenic in situ hybridisation and fluorescence in situ hybridisation analysis

Immunohistochemistry for CD44 was performed on the TMA cohort using the pan anti-human CD44 monoclonal antibody clone DF1485 (Dako, Cambridgeshire, UK) at a dilution of 1:20. Detection was carried out using the Dako EnVision system with 3,3'-diaminobenzidine as chromogen. Only membranous with or without cytoplasmic reactivity was considered specific. Expression of CD44 was determined using the Allred semi-quantitative scoring system: Intensity of staining was scored as 0 (no staining at high magnification), 1 (weak staining only visible at high magnification), 2 (moderate staining readily visible at low magnification), 3 (strong staining visible at low magnification). The proportion of tumours showing positive staining was scored as follows: 0 (0%), 1 (<1%), 2 (1–10%), 3 (11–33%), 4 (34–66%), 5 (67–100%). Intensity and proportion of staining were added and cases classified as negative (0–2), low expression (3–5) and high expression (6–8). CD44 immunohistochemical analysis was performed by two observers (CM & JRF) blinded to the results of other immunohistochemical markers, *CD44* CISH and patients' outcome.

CISH for *CD44* was performed on the TMA cohort utilising in house, biotin labelled probes as previously described [31]. These probes were generated using the BAC clones RP11-30D23 and RP11-790K21, which encompass the region between 34.99 and 35.25 Mb on chromosome 11. The specificity of the probe and its genomic position were confirmed by SP6/T7 end pair sequencing and fluorescence in situ hybridisation (FISH) mapping using normal lymphocyte metaphase spreads as previously described [31]. For analysis, signals were counted in at least 60 non-overlapping nuclei of morphologically unequivocal cells. Cases were considered amplified by CISH if >50% of neoplastic cells harboured large signal clusters or >5 signals/nucleus or large gene clusters, as previously described [22, 32]. CISH analysis was performed by two observers (CM & JRF) blinded to the results of CD44 and other immunohistochemical markers, and patients' outcome.

FISH analysis was performed on interphase and metaphase chromosomes from breast carcinoma cell lines. A

CD44 locus specific FISH probe BAC RP11-79OK21 was labelled with digoxigenin as previously described [31] and detected with anti-digoxigenin-fluorescein (Roche). The biotinylated chromosome 11 centromere was labelled with Cy3-streptavidin (Sigma). Nuclei were counterstained with DAPI (4,6-diamino-2-phenylindole). Metaphase images were captured using a Zeiss Axioplan2 microscope, equipped with Applied Imaging CCD camera and Cytovision software, version 2.81 (Applied Imaging).

#### RT-PCR and splice variant analysis

Total RNA of cell lines was isolated using the RNeasy Kit (Qiagen, Crawley, UK), and reverse transcribed with the Omniscript RT Kit using oligo dT primers (Qiagen). For identification of CD44 isoforms, variant sequences were amplified with CD44p1 and CD44p2 primers, which are located outside the variant region, as previously described [33]. Amplified sequences were subcloned using the TOPO TA cloning Kit (Invitrogen) and identified by sequencing. RNA from tumour samples was isolated using Trizol according to manufactures instructions (Invitrogen, UK) reverse transcribed and amplified with SuperScript III One step RT-PCR System (Invitrogen) using CD44p1 and CD44p2 primers. Amplified products were subcloned and sequenced as described above.

#### Quantitative RT-PCR

Reverse transcription of 75 ng total RNA was performed as described above. qRT-PCR was performed with Taqman Gene Expression Assays (Applied Biosystems, Drive Foster City, CA, USA) using human CD44 (Hs00153304\_m1), human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (4310884E) and  $\beta$ -actin (4310881E) probes on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Warrington, UK). Data were collected for each cell line in triplicates for at least two independent RNA isolations. Data were analysed with the SDS 2.2.1 software. Fold change is represented by relative quantification (RQ) units, error bars indicate calculated RQmin and RQmax values, based on a confidence level of 95%.

#### siRNA treatment, proliferation and survival assays

A single CD44 siRNA 5-GUAUGACACAUAUUGCUUCdT-3 and 5-GAAGCAAUAUGUGUCAUACdT-3 [34] (Dharmacon, Cramlington, UK) was used for transfection of all cell lines with Oligofectamine Reagent (Invitrogen) according to the manufacturers' instructions at a final concentration of 100 nM. A non-targeting siRNA (On-Target plus control siRNA; Dharmacon) and mock transfections were used as controls. To assess proliferation, cells were

seeded 24 h after siRNA transfection into 96 well plates at  $2 \times 10^3$  (HCC1500:  $1 \times 10^4$ ) cells per well in medium containing 0.5 or 10% FCS in triplicates. At different time points, cell number was quantified by CellTiter-Blue (Promega, Southampton, UK). Proliferation was estimated relative to time 0 (48 h after transfection). For survival assays, cells were seeded 24 h after siRNA transfection into 96 well plates at  $1 \times 10^4$  (HCC 1500:  $5 \times 10^4$ ) cells per well in triplicate. 48 h after transfection the medium was aspirated and replaced with medium containing doxorubicin (Sigma). Survival of treated cells relative to untreated cells was quantified using CellTiter-Blue (Promega) 72 h after drug treatment.

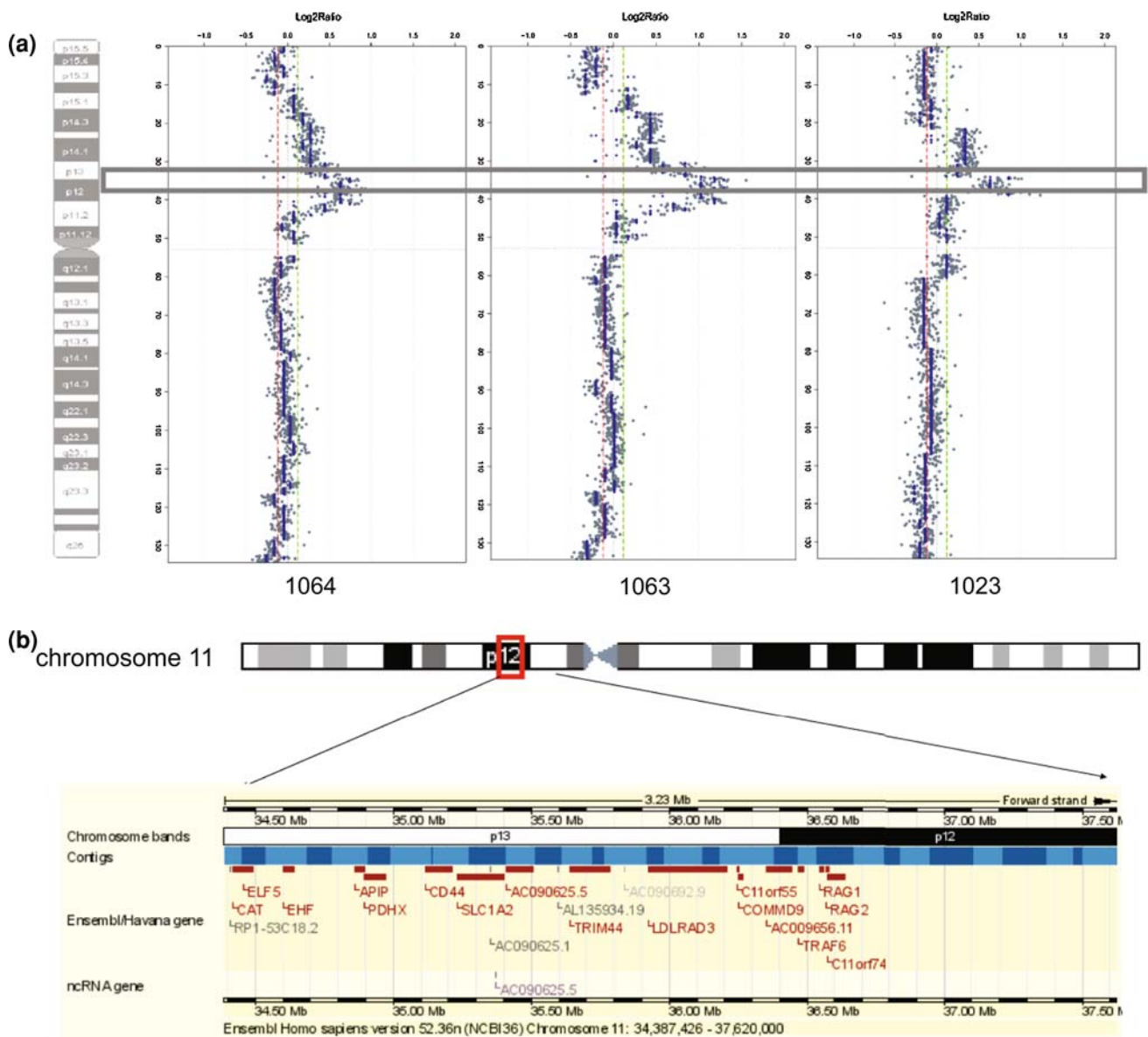
#### Statistical analysis

The Statview version 5 statistical package was used for all statistical analysis of the TMA series. Correlations between categorical variables were performed using the Chi-square test and Fisher's exact test. Correlations between continuous and categorical variables were performed with the Mann-Whitney U test. Disease-free survival (DFS) was expressed as the number of months from diagnosis to the occurrence of local recurrence in the form of ductal carcinoma in situ and/or invasive breast cancer. Metastasis-free survival (MFS) was expressed as the number of months from diagnosis to the occurrence of distant relapse. Breast cancer specific survival (BCSS) was expressed as the number of months from diagnosis to the occurrence of an event (disease-related death). Cumulative survival probabilities were calculated using the Kaplan-Meier method. Differences between survival rates were tested with the Log-Rank test. Correlations between CD44 mRNA expression levels, 11p13 amplification and molecular subtype were performed using two tailed unpaired *t*-test. All tests were two-tailed, with a confidence interval of 95%.

## Results

### Amplification of *CD44* in breast cancers

Out of the 100 GIII-IDC-NSTs analysed with aCGH, 25 were classified as basal-like, 25 as HER2, 45 as luminal and 5 of indeterminate phenotype using a previously validated surrogate for the breast cancer molecular subgroups [35]. The detailed analysis of the genetic profiles of these tumours is reported elsewhere [18]. aCGH analysis revealed amplifications of 11p13-p12 (34.38–37.62 Mb) in 3/100 (3%) tumours (Fig. 1a), with the minimal region of amplification mapping to 34.38–37.62 Mb. According to Ensembl release 51 (<http://www.ensembl.org>), this region incorporated 19



**Fig. 1** 11p12-p13 amplification in primary grade III breast cancers. **a** Representative chromosome 11 plots of primary grade III invasive ductal carcinomas of no special type with 11p12-p13 amplification (tumours 1064, 1063, 1023). Log2 ratios are plotted on the Y axis against each clone according to genomic location on the X axis.

genes *ELF5*, *CAT*, *EHF*, *APIP*, *PDHX*, *CD44*, *SLC1A2*, *AC090625.1*, *AC090625.5*, *FJX1*, *TRIM44*, *AC090692.9*, *C11orf55*, *TRAF6*, *COMMD9*, *RAG1*, *AC009656.11*, *RAG2* and *C17ORF74* (Fig. 1b).

In this cohort, amplification at this locus was seen in 3/29 triple negative tumours (ER<sup>-</sup>, PgR<sup>-</sup>, HER2<sup>-</sup>), and 0/71 tumours of non-triple negative phenotype ( $P = 0.0226$ , Fishers-exact test). One amplified tumour also displayed positivity for basal marker expression and all had a ‘saw-tooth’ pattern of genomic alterations [30]. No other significant associations between amplification of 11p13 and

AWS-smoothed ratios are co-plotted (blue). The box highlights the common region of amplification at 11p13. **b** Detail of the genomic position and genes mapping to the smallest region of amplification of 11p12-p13 obtained from <http://www.ensembl.org>

other clinicopathological or immunohistochemical parameters were found. No material from the cohort subjected to aCGH was available for CD44 immunohistochemical assessment.

To determine the prevalence of 11p13 amplification in an independent cohort of breast cancers, we generated CISH probes mapping to the smallest region of amplification, specifically to *CD44*, and applied these to a TMA containing 245 primary breast cancers. *CD44* gene amplification was observed in 3 out of 125 (2.4%) interpretable samples (Fig. 2). All cases harbouring *CD44* amplification

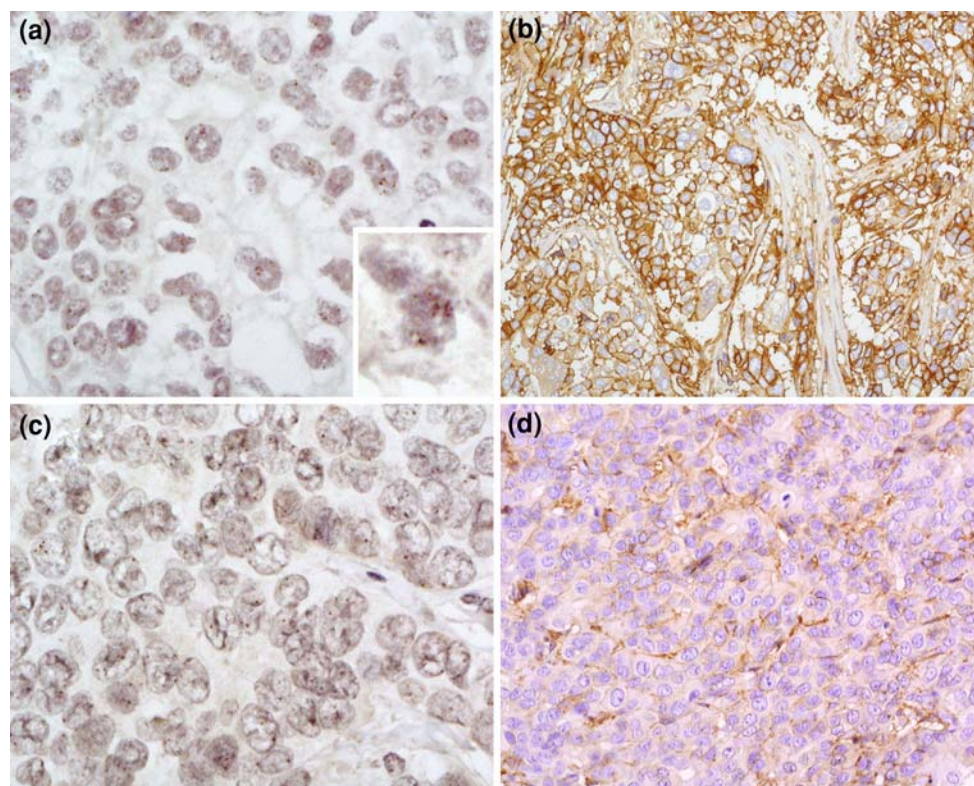
were of histological grade III; two were invasive ductal carcinomas and one was a pleomorphic lobular carcinoma. In this cohort, we could not confirm the association between triple negative phenotype and *CD44* amplification; of the three amplified cases, two were of luminal phenotype (ER+, PR-, HER2-) and one was of basal-like phenotype (ER-, PR-, HER2-, and positive for EGFR and basal keratins). *CD44* amplification was not significantly correlated with any clinico-pathological features of the tumours after Bonferroni correction for multiple comparisons. No correlations with disease-free, metastasis-free or overall survival were found in the whole series or when exploratory analysis was performed in ER+, ER-, lymph node positive and lymph node negative subgroups (data not shown). It should be noted, however, that owing to the small sample size and the low prevalence of *CD44* amplification, type II or  $\beta$  errors cannot be ruled out.

Immunohistochemical analysis demonstrated that all cases with *CD44* amplification as determined by CISH displayed high levels of CD44 expression (Table 1; Fig. 2). Within the whole cohort, CD44 expression was significantly associated with lack of ER and PR, lack or low levels of cyclin D1 expression and with expression of EGFR, Ck 5/6, Ck 14 and Ck 17, high proliferation rates as

defined by Ki67 (MIB1) and topoisomerase II $\alpha$ , and p53 nuclear expression. Furthermore, high levels of CD44 expression were preferentially found in tumours of basal-like and triple negative phenotype (Table 1). All cases with *CD44* gene amplification expressed high levels of CD44 protein expression. Univariate survival analysis failed to reveal any association between CD44 expression and disease-free, metastasis-free or overall survival (data not shown).

#### Amplification of *CD44* in breast cancer cell lines

Re-analysis of publicly available aCGH data from 50 breast cancer cell lines using a 1 Mb BAC array, revealed the presence of an 11p13 amplification in four basal-like breast cancer cell lines (SUM149, MDA-MB-468, HCC1954 and HCC1500) [36]. By performing aCGH of these cell lines on our 32 K tiling path BAC array platform (resolution of 50 Kb), amplification of this genomic locus was confirmed in SUM149 and MDA-MB-468 cells. HCC1954 cells were found not to be amplified at the CD44 locus but harboured a majority of tetraploid cells. HCC1500 harboured amplification of 11p13 but this amplified region lay proximal (centromeric) to the *CD44*



**Fig. 2** *CD44* gene copy number and protein expression in invasive breast cancer. **a, b** Invasive ductal carcinoma displaying *CD44* gene amplification as defined by chromogenic in situ hybridisation (**a**), and strong, membranous and cytoplasmic immunohistochemical

expression (**b**). Note the presence of small (**a**) and large (**a—*inset***) *CD44* gene clusters. **c, d** Invasive ductal carcinoma with one to two *CD44* gene copies per nucleus of neoplastic cells (**c**) displaying low levels of *CD44* membranous protein expression (**d**)

**Table 1** Correlations between CD44 expression, clinicopathological parameters and immunohistochemical markers in 245 invasive breast carcinomas

Parameter	<i>N</i>	CD44–	CD44 low	CD44 high	<i>P</i> value
Size (mm)	176	35.43 ± 26.78	23.43 ± 14.13	24.89 ± 15.06	<b>0.0109*</b>
Size	176				0.1095**
T1		7	42	43	
T2		37	22	13	
T3		5	4	3	
Grade	173				0.6932**
I		2	8	5	
II		8	19	22	
III		13	41	55	
Type	175				0.9613**
IDC		15	50	62	
ILC		4	10	13	
Mixed		3	6	4	
Other		1	3	4	
LVI	176				0.2758**
–		6	18	32	
+		17	50	53	
LN metastasis	171				0.5469**
–		8	21	34	
+		13	45	50	
ER	173				<b>0.0358**</b>
–		4	8	25	
+		19	57	60	
PR	173				<b>0.0144**</b>
–		4	11	31	
+		19	54	54	
HER2	172				0.7116**
–		21	54	73	
+		2	10	12	
HER2 CISH	167				0.6212**
Not Amp		20	53	70	
Amp		2	11	11	
Triple negative	173				<b>0.0034**</b>
No		21	62	65	
Yes		2	3	20	
EGFR	176				<b>0.0124**</b>
–		22	66	71	
+		1	2	14	
Ck 14	176				0.0599**
–		22	66	74	
+		11	2	1	
Ck 5/6	170				<b>0.0394**</b>
–		22	61	70	
+		0	4	13	
Ck 17	174				<b>0.0119**</b>
–		22	64	66	
+		1	4	17	

**Table 1** continued

Parameter	<i>N</i>	CD44–	CD44 low	CD44 high	<i>P</i> value
Basal markers	176				<b>0.0026**</b>
–		22	62	62	
+		1	6	23	
Caveolin 1	176				<b>0.0071**</b>
–		22	66	70	
+		1	2	15	
Caveolin 2	159				0.2231**
–		20	60	68	
+		1	2	8	
Nielsen groups	164				<b>0.0030**</b>
Basal		1	2	20	
Luminal		18	49	50	
HER2		2	11	11	
P53	163				<b>0.0079**</b>
–		19	50	46	
+		4	12	32	
MIB-1 (%)	163				<b>0.0113**</b>
<10		10	29	26	
10–30		11	31	31	
>30		2	3	20	
FOXA1	142				0.2116**
–		3	10	21	
+		14	46	48	
Cyclin D1	165				<b>0.0132**</b>
Low		2	2	16	
Intermediate		2	11	17	
High		19	49	47	
<i>CCND1</i> CISH	154				0.6651**
Not Amp		15	49	62	
Amp		2	10	16	
Topoisomerase II $\alpha$	158				<b>0.0491**</b>
Low		14	35	32	
High		6	27	44	
<i>TOP2A</i> CISH	167				0.2690**
Not Amp		22	56	75	
Amp		0	7	7	
<i>MYC</i> CISH	148				0.3090**
Not Amp		18	49	65	
Amp		1	9	6	

*Amp* amplified, *CISH* chromogenic in situ hybridisation, *Ck* cytokeratin, *EGFR* epidermal growth factor receptor, *ER* oestrogen receptor, *LN* lymph node, *LVI* lymphovascular invasion, *N* number, *Nielsen groups* immunophenotypic groups defined based upon the expression of ER, HER2, Ck 5/6 and EGFR, *PR* progesterone receptor, *triple negative* ER, PR and HER2 negative cancers as defined in Tan et al. [21]

\* Two-tailed *t*-test;

\*\* Chi-square test

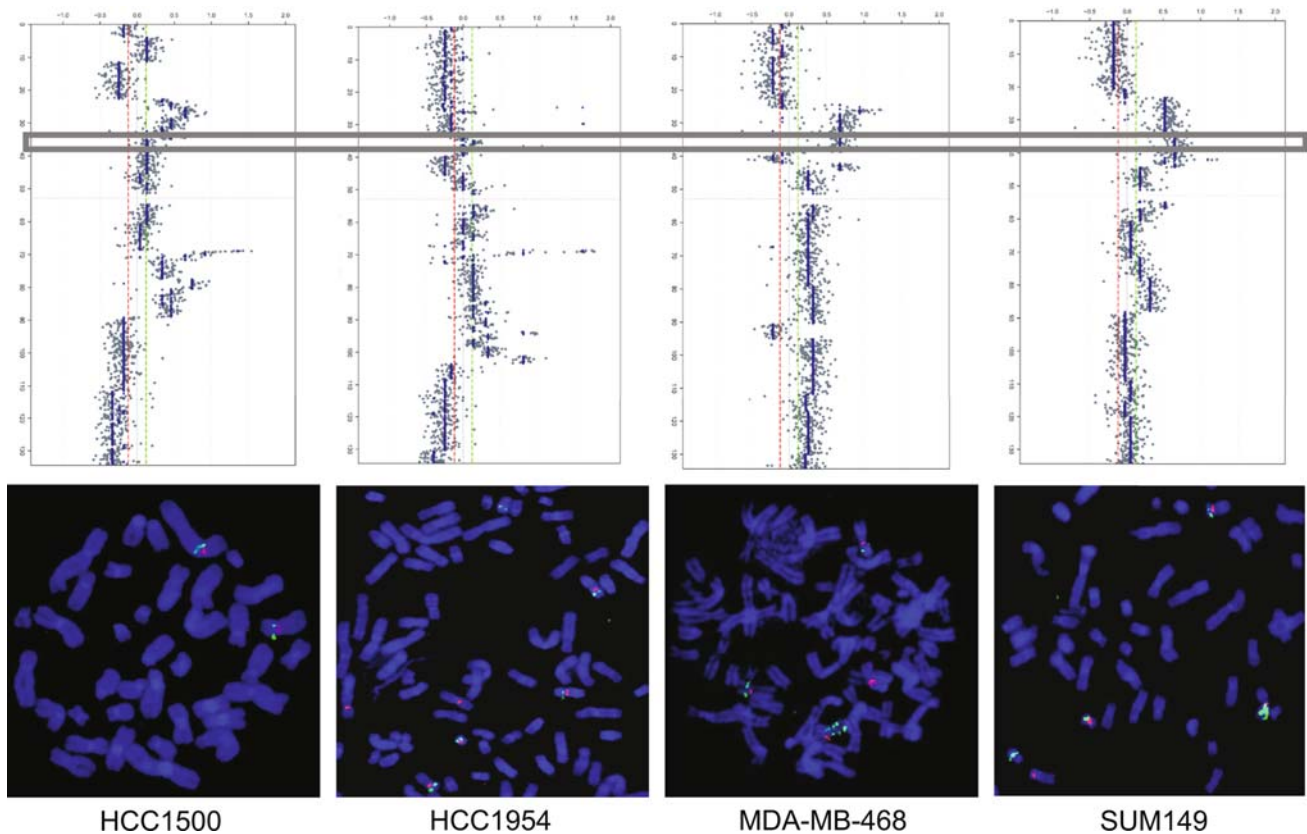
Statistically significant *P* values are marked in bold

gene. These data were further verified by dual colour metaphase FISH analysis (Fig. 3). Multiple copies of *CD44* were detected in SUM149 and MDA-MB-468 cells, in the form of clusters of signals indicative of HSR's (homologously staining regions), whereas no copy number gains or amplification of 11p13 were detected in control basal-like breast cancer cell lines HCC1500 or HCC1954 (Fig. 3) or other breast cancer cells lines examined (HS578T, MCF7, BT474, SKBR3, MDA-MB-231, ZR75-1; data not shown; range of the 11p13:CEP11 ratios 0.81-1).

*CD44* amplification is associated with high levels of CD44 mRNA expression

CD44 mRNA levels were assessed by real-time quantitative PCR (qRT-PCR) in a panel of human breast cancer cell lines (Fig. 4a) and correlated with 11p13 gene amplification and the molecular phenotype. Cell lines with 11p13 amplification displayed significantly higher levels of CD44 mRNA expression ( $3.1480 \pm 0.6536$ ) than cell lines lacking amplification of this region ( $1.0018 \pm 0.2978$ , *t*-test two-tailed





**Fig. 3** 11p12-p13 amplification in breast cancer cell lines. Representative chromosome 11 plots and metaphase spreads of breast cancer cell lines lacking (HCC1500 and HCC1954) or harbouring (MDA-MB-468 and SUM149) 11p12-p13 amplification. Log<sub>2</sub> ratios are plotted on the X axis against each clone according to genomic location on the Y axis. AWS-smoothed ratios are co-plotted (blue).

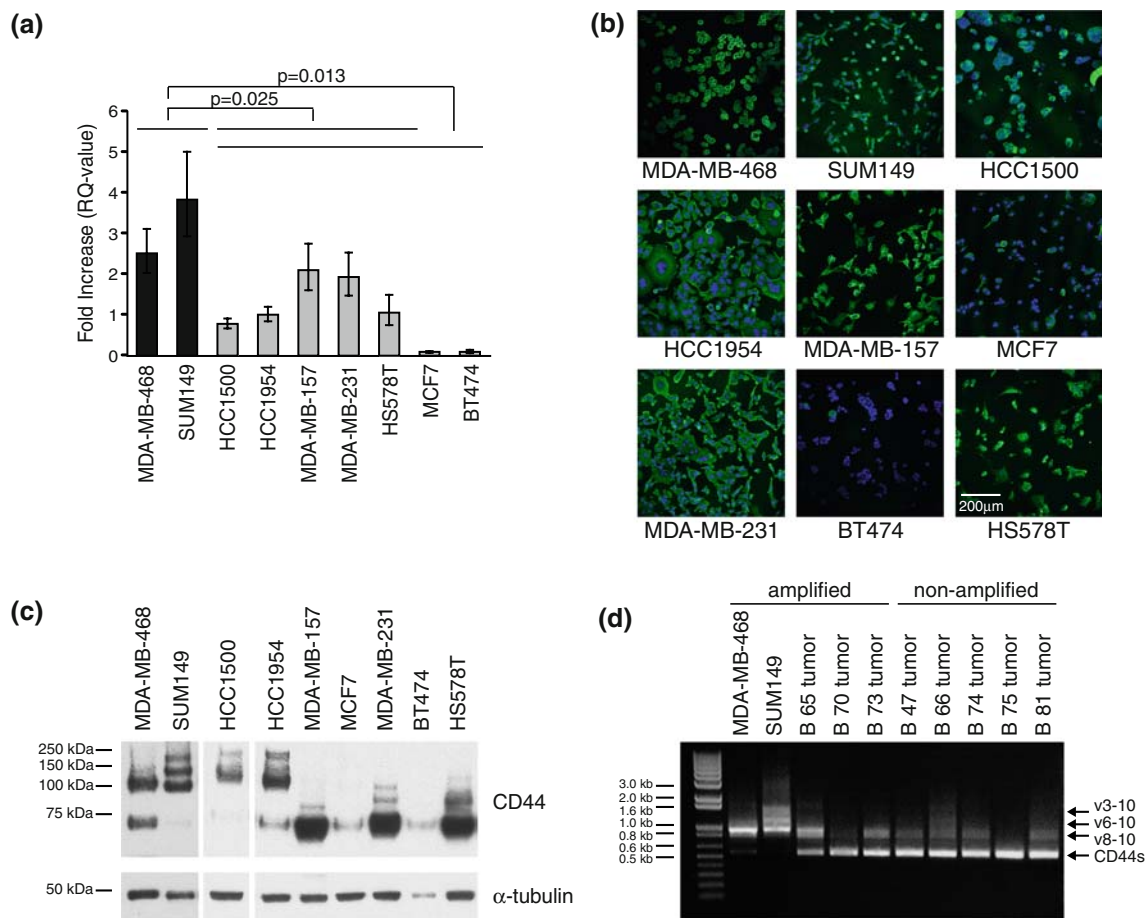
The box highlights the common region of amplification at 11p13. In the metaphase spreads, fluorescence in situ hybridisation (FISH) was performed with probes for chromosome 11 centromere (red) and *CD44* (green). A perfect agreement between array CGH and FISH results was observed. Note the presence of multiple *CD44* signals in MDA-MB-468 and SUM149 cells

$P$  value = 0.013). *CD44* mRNA levels were significantly higher in basal-like ( $2.0124 \pm 0.4475$ ) than in luminal breast cancer cell lines ( $0.0921 \pm 0.0021$ ,  $t$ -test two-tailed  $P$  value = 0.008). Given the association between *CD44* expression and basal-like phenotype, we tested whether the expression of *CD44* mRNA would be higher in basal-like cell lines with 11p13 amplification than in cell lines lacking amplification of this region: basal-like cell lines with 11p13 amplification displayed expression levels significantly higher ( $3.1480 \pm 0.6536$ ) than the remaining basal-like cell lines ( $1.3656 \pm 0.2653$ ,  $t$ -test two-tailed  $P$  value = 0.025). Equivalent results were obtained when *CD44* mRNA levels were normalised to *GAPDH* (data not shown).

*CD44* protein levels were assessed by Western blotting and immunofluorescence (Fig. 4b–c). As previously reported [14] and in agreement with the mRNA levels, negligible *CD44* protein was detected in the luminal breast cancer cell lines, MCF7 and BT474. High levels of *CD44* protein were observed in the two *CD44* amplified basal-like cell lines, MDA-MB-468 and SUM149, but these levels were not

notably higher than in the non-amplified basal-like cell lines HCC1500, MDA-MB-157, MDA-MB-231 and HS578T. Expression of alternatively spliced variant *CD44* isoforms was observed in the two amplified cell lines, MDA-MB-468 and SUM149, but also in the non-amplified cells lines, HCC1500 and HCC1954. All other cell lines expressed only the standard *CD44*s, which contains none of the alternatively splice exons. All cell lines expressing variant *CD44* isoforms showed decreased expression of the standard *CD44*s isoform (Fig. 4c).

To characterise the *CD44* isoform expression, mRNA from the cell lines was amplified by RT-PCR using primers located outside the variant regions (Fig. 4d). Consistent with the Western blot data (Fig. 4c), sequencing of the RT-PCR products demonstrated that in addition to the standard *CD44*s isoform, the *CD44*v8-10 isoform was prominently expressed by the HCC1954, HCC1500, MDA-MB-468 and SUM149 cell lines, with the SUM149 cells also showing expressing of the *CD44*v3-10 and *CD44*v6-10 isoforms. As MDA-MB-468 and SUM149



**Fig. 4** CD44 expression in amplified and non-amplified cell lines. **a** Real time quantitative RT-PCR analysis of CD44 mRNA levels in breast cancer cell lines. Fold increase is shown relative to HCC1954 cells.  $\beta$ -actin served as endogenous control. *Black bars*, amplified basal-like cell lines; *grey bars*, non-amplified basal-like cell lines, *white bars*, non-amplified luminal cell lines. **b** Cells were fixed and stained for CD44 with E1/2 antibody followed by Alexa488 goat anti-

mouse Ig. Nuclei were counterstained with DAPI. *Scale bar*, 200  $\mu$ m. **c** Cells were lysed and subject to Western blot analysis with the E1/2 anti-CD44 antibody which recognises all CD44 isoforms. Blotting for  $\alpha$ -tubulin served as loading control. **d** End point RT-PCR of amplified cell lines and primary breast cancer samples. Isoforms indicated by *arrows* were identified by sequencing

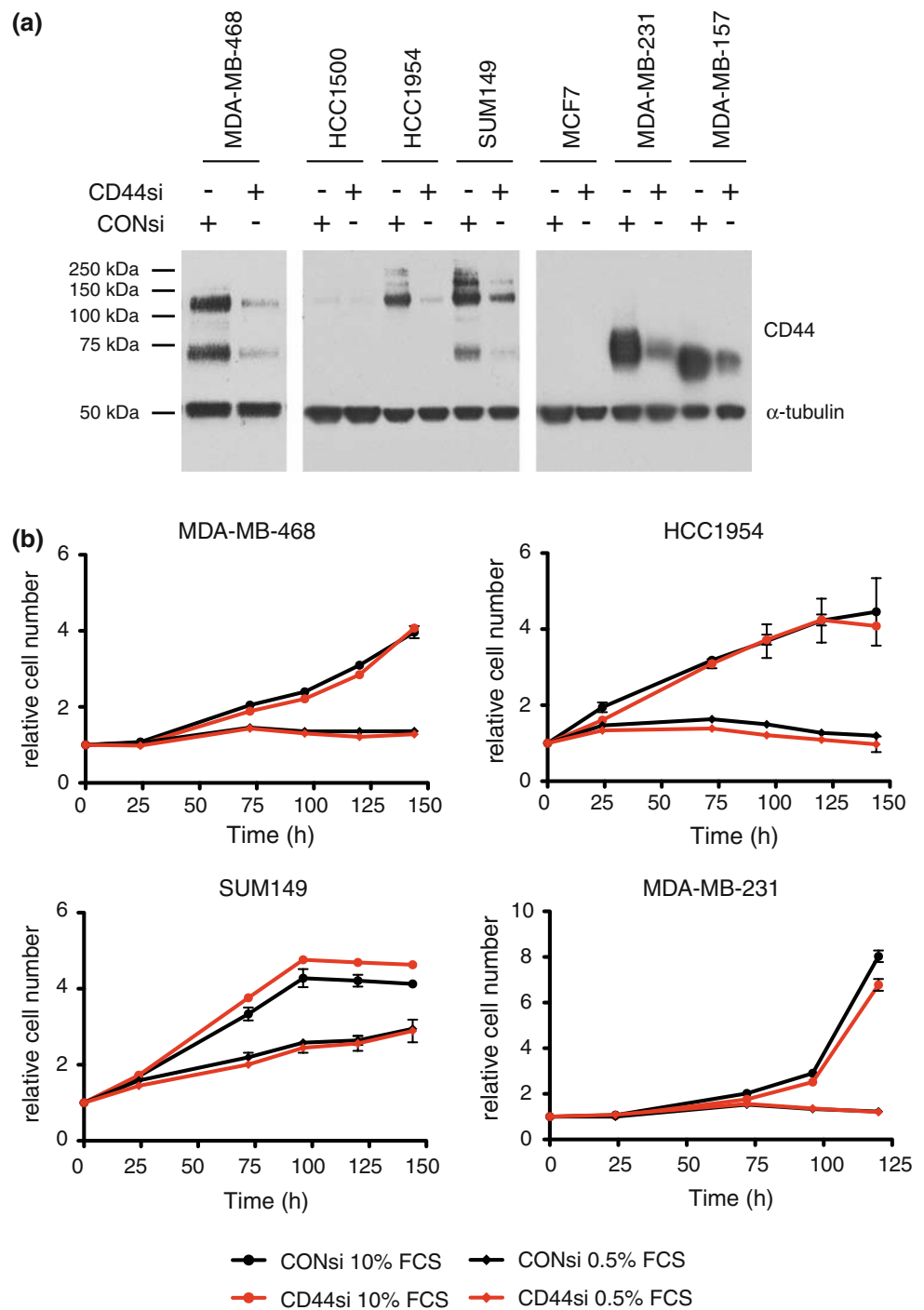
cells, the two cell lines carrying the amplification, showed alternative splicing, we tested the possibility that amplification deregulates the splicing control of CD44. The three primary breast tumours identified by aCGH to harbour amplification of *CD44* (Fig. 1) and five control tumours were analysed for expression of splice variants by RT-PCR. All analysed samples showed expression of CD44s and/or CD44v8-10 with no clear differences between tumours carrying the *CD44* amplification and those with a non-amplified locus (Fig. 4d).

Cell lines with 11p13 amplification are not dependent on CD44 expression for proliferation or survival

Given that primary tumours and cell lines harbouring 11p13 amplification consistently expressed high levels of CD44 and that genes overexpressed when amplified are

potential amplicon drivers, we hypothesised that *CD44* would be the driver of 11p13 amplification. To test this hypothesis, we performed siRNA-mediated downregulation of CD44 in a panel of breast cancer cell lines and analysed the proliferative and survival potential. Transfection with CD44 siRNA resulted in a substantial downregulation of CD44 expression as revealed by western blotting and immunohistochemistry (Fig. 5a). Of note, these siRNAs were directed against sequences in the non-variant CD44 backbone and efficiently resulted in downregulation of both standard and alternatively spliced isoforms. Effective downregulation was achieved 2 days after transfection and was stable for 4 days (data not shown). As shown in Fig. 5b, proliferation of MDA-MB-468, SUM149, HCC1954 and MDA-MB-231 cells was unaffected by CD44 downregulation both in high and low serum conditions. Survival upon drug treatment was analysed after

**Fig. 5** Downregulation of CD44 expression has no differential impact on the proliferation of cell lines with or without the *CD44* containing amplicon. Cells were transfected with CD44 siRNA oligonucleotides (CD44si) or non-targeting control siRNA oligonucleotides (CONsi). **a** Western blot analysis demonstrating efficient downregulation of all CD44 isoforms in all cell lines tested.  $\alpha$ -tubulin served as loading control. **b** Proliferation was analysed for 6 days under high (10%) or low (0.5%) serum conditions and is presented as relative fold increase. The two basal-like cell lines carrying the amplicon (MDA-MB-468, SUM149) and two non-amplified basal-like cell lines (HCC1954, MDA-MB-231) are shown

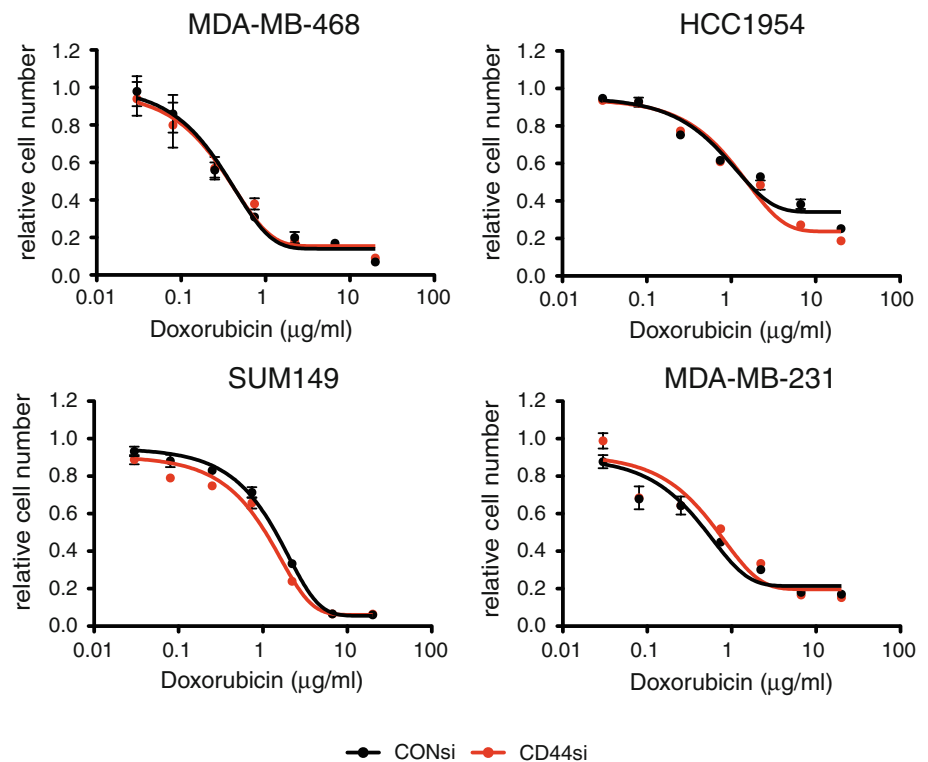


3 days of doxorubicin treatment in the same cell lines. Again, no CD44 dependent differences in susceptibility were observed in the cell lines tested (Fig. 6). Similar results were obtained for all the other cell lines examined (data not shown). Taken together, these results provide strong circumstantial evidence to suggest that CD44 is not the driver of the 11p13 amplicon in breast cancers.

## Discussion

The development of high-resolution aCGH platforms have allowed the rapid identification in both tumour tissue and cancer cell lines of regions of chromosomal amplification, harbouring potential oncogenes, and chromosomal deletions, harbouring potential tumour suppressor genes [37].

**Fig. 6** Downregulation of CD44 expression has no differential impact on the sensitivity to drug treatment of cell lines with or without the CD44 containing amplicon. Cells were transfected with CD44 siRNA (CD44si) or a non-targeting control siRNA (CONsi) oligonucleotides. Survival was monitored after 3 days of doxorubicin treatment at stated concentrations. The two basal-like cell lines carrying the amplicon (MDA-MB-468, SUM149) and two non-amplified basal-like cell lines (HCC1954, MDA-MB-231) are shown



Here we describe the identification of a subset of breast cancers and cell lines harbouring amplification at 11p13 in which the minimal region of amplification encompasses 19 genes including *CD44* (Fig. 1b). Of these the presence of CD44 was intriguing given the wealth of information on a role for CD44 in tumour progression and metastasis in a variety of cancer types [1, 2] and more recently in the identification of CD44 as a marker of cancer stem/progenitor cells [38]. Amplification of 11p12-p13 has been identified in head and neck squamous cell carcinoma and gastric carcinoma cell lines [39, 40] and the presence of this amplification was shown to be associated with significantly increased levels of CD44 expression. Consistent with these observations, here we provide the first direct evidence that CD44 is consistently expressed at high levels in primary breast cancers and breast cancer cell lines harbouring 11p13 amplification. It should be noted, however, that in a way akin to *EGFR*, *CAVI*, *MYC*, *CCND1* [32, 36, 41–43], high level expression of CD44 was more pervasive than gene amplification. In agreement with previous studies [14, 44], we detected high levels of CD44 expression, as determined by qRT-PCR and Western blotting, in all basal-like cell lines examined and low or negligible levels of expression in the luminal cell lines. Moreover, by staining a large cohort of invasive breast cancers we found a significant correlation between expression of CD44 and basal-like breast cancers (Table 1). Despite the association with basal-like phenotype, CD44 expression did not show a correlation with the outcome of breast cancer patients.

The mechanisms that regulate CD44 splicing are not fully understood but it is clear that in normal cells, splicing is regulated by tissue specific factors. Cells of mesenchymal origin predominantly express the standard CD44 isoform, CD44s, in which all of the variant exons are spliced out whereas normal epithelial cells frequently show expression of the variant CD44 isoforms, in particular CD44v8-10, CD44v3-10 and CD44v3,v8-10 [2]. It is also well established that the pattern of CD44 splice variant expression is frequently altered in tumour cells, with expression of variant exons v6 and v7 being commonly observed. Consequently we explored in this study whether amplification of the *CD44* locus was associated with a different pattern of CD44 isoform expression. It was notable that the two amplified cell lines did indeed express high levels of variant CD44 isoforms as detected by sequencing and Western blotting, however, again this variant expression was not restricted to the amplified cell lines. Similarly, no differences in *CD44* splicing were detected between amplified and non-amplified primary breast cancers. It was of interest to note, that the MDA-MB-157, MDA-MB-231 and HS578T cell lines predominantly expressed CD44s and these cell lines are all placed into the mesenchymal group based on expression profiling [44].

A key aspect of genomic analysis is to identify regions of amplification which contain oncogenic drivers and which are therefore potential therapeutic targets. In breast cancer, this is best exemplified by the *HER2* (*ErbB2*)

amplification found in 18–20% of breast cancer patients and the clinical use of the HER2 targeted treatments, trastuzumab and lapatinib [45]. However, the identification of a region of genomic amplification poses two major problems. First, it is necessary to identify which of the co-amplified genes is the oncogenic driver. Second, owing to the inherent genomic instability of cancer cells, cancers may also contain somatically acquired genomic amplifications that do not necessarily confer a selective advantage. We and others have demonstrated that drivers of specific amplicons are consistently expressed at high levels in the presence of amplification of their loci and that amplicons often harbour more than one driver [18, 46, 47]. For instance, RNA interference studies have demonstrated that cell lines harbouring 17q12 amplification, are not only dependent on HER2 expression, but also on the expression of GRB7 and STARD3 [47]. Given the longstanding interest in the role of CD44 in tumorigenesis and as a potential cancer stem/progenitor cell marker, together with our observation that CD44 was consistently expressed at high levels when amplified, we examined whether cells harbouring *CD44* gene amplification were dependent on CD44 expression for their proliferation and survival. We found no evidence that CD44 expression was required for the proliferation or survival of cells with 11p13 amplification, strongly suggesting that CD44 is not the driver of this amplicon. Further studies will be required to establish whether cell lines and tumours harbouring this region of amplification are dependent on the expression of any of the other 18 genes.

## Conclusions

A subset of breast cancers and breast cancer cell lines harbour an amplification at 11p13 encompassing the *CD44* gene. CD44 is consistently expressed at high levels in primary cancers and cell lines with *CD44* amplification. Unlike many other genes consistently overexpressed when amplified (e.g. *HER2*, *EGFR*, *PPM1D*) [18], CD44 expression is not required for the proliferation or survival of cells with 11p13 amplification. Together these data provide strong circumstantial evidence to suggest that CD44 is unlikely to be the driver of 11p13 amplification.

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