Characterization of Kelch domain-containing protein 7B in breast tumours and breast cancer cell lines

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Abstract. Adenocarcinomas exhibit great heterogeneity, with many genetic and epigenetic alterations. The Kelch domain-containing protein 7B (KLHDC7B) has recently been identified as epigenetically modified and upregulated in breast cancer. The potential reversibility of epigenetic states offers exciting possibilities for novel cancer diagnostics and drugs. However, to properly evaluate specific inhibitors, the role of KLHDC7B in the development and progression of breast cancer should be established. With that objective in mind, the present study investigated a series of human breast tumours and correlated their clinicopathology, according to the Elston-Ellis modification of the Scarff-Bloom-Richardson (SBR) grading system, with KLHDC7B mRNA expression, analysed using quantitative PCR (qPCR). The results revealed that KLHDC7B was significantly upregulated in grade 3 tumours, and that KLHDC7B expression varied according to the tumour grade and the individual, being downregulated in well-differentiated and moderately-differentiated tumours (grade 1-2) and upregulated in poorly-differentiated tumours (grade 3). Immunohistochemical staining revealed that ductal tumours and tumours with a higher percentage of Ki67 positive cells showed the highest levels of KLHDC7B. Receptor expression, HER, p53 status, presence of metastasis, and vascular invasion showed no association with KLHDC7B expression. Previous studies have proposed KLHDC7B as an epigenetic marker of breast cancer. We propose that KLHDC7B should be used as a marker for poorly-differentiated tumours only; use of KLHDC7B without considering tumour grade could lead to an inaccurate diagnosis. Finally, we suggest the appropriate breast cancer cell lines to use to determine the functions of KLHDC7B. KLHDC7B expression was tested in the non-tumour cell line MCF-10A and in the breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-468, using qPCR and western blotting. The results revealed that all tested cancer cell lines overexpressed KLHDC7B mRNA, but MDA-MB-468 exhibited a much lower level of protein expression relative to mRNA. Although the breast cancer cell lines used may be appropriate for studying KLHDC7B epigenetic status, MDA-MB-468 should be excluded from functional experiments.

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Introduction

Breast cancer is the second most common cancer worldwide and the most frequent cancer among women (1). One in 8 women in Europe will develop breast cancer before the age of 85 (2). The highest prevalence is found in northern and western European countries, suggesting a relation to environmental factors (3,4). Metastatic breast cancer is the second leading cause of cancer-related fatality in women: It has a five-year relative survival rate of 23%, compared with 99% for non-metastatic breast cancer (5). Breast tumours are classified according to the Elston-Ellis modification of the Scarff-Bloom-Richardson (SBR) grading system (also known as the Nottingham grading system) (6). This system grades tumours according to their differentiation, from well differentiated (grade 1), to moderately differentiated (grade 2) or poorly differentiated (grade 3). This histological scale is used as a prognostic predictor in patients with breast cancer, as tumour grade has a positive correlation with metastasis and risk of recurrence.

Regulation of signal transduction and stress response is critical to maintain cellular homeostasis. Cell signals in response to stress can result in growth arrest and elimination of damaged or premalignant cells. During cancer development, signalling pathways are often impaired due to dysregulation of gene expression or aberrant signal transduction, resulting in the hallmarks of cancer (7-13). Gene expression is largely regulated by epigenetic changes, including DNA methylation and histone modification (14). More than 50% of human cancers harbour mutations in enzymes involved in chromatin organization (15). One of the biggest challenges in cancer research is to understand how defects arise during disease progression. This is likely to become increasingly important to detect and validate biomarkers for tumour gradings and subtypes that may help guide treatment decisions (16).

One gene that has recently been found to be involved in breast and ovarian cancer and lymph node metastasis in cervical cancer is the Kelch domain-containing protein 7B (KLHDC7B) gene (13,17-19). It has been identified as hypermethylated and upregulated in breast cancer (17), and, when associated with alternative splicing events, may be involved in the development and progression of cervical squamous cell carcinoma (CSCC) (20).

The KLHDC7B gene (Hs.137007) (21) comprises a single exon, located on human chromosome 22q13.33 (22,23). In 50 paired samples of breast cancer tissue and adjacent normal tissue, the methylation level of the 14 CpG sites at the promoter region of the gene was higher in cancerous tissue (72-93%) than in normal tissue (31-83%) (17). A clear relationship between high methylation levels and upregulated expression was also observed in cultured breast cell lines. For instance, MCF-7 (90-100%) and MDA-MB-468 (100%) cancer cell lines had higher methylation at the 14 CpGs and higher gene expression than BT549 (20-90%) and 184B5 (10-100%) cell lines (17).

Numerous reports have described an association between hypermethylation of individual genes and clinical prognosis for various types of cancer, and individual methylation markers have previously been linked to breast cancer metastasis (24). DNA methylation is generally associated with gene downregulation. However, some genes, including survivin (25), the glycoprotein hormone alpha-subunit (26), and KLHDC7B, have been found to be upregulated when CpG sites are hypermethylated (17).

The potential reversibility of epigenetic status offers exciting opportunities for cancer treatments, and targeting methylation represents the third wave of anticancer drug development (24). DNA methyltransferases currently represent one of the major drug targets, and new drugs are expected to be added in the near future (24,27,28).

The KLHDC7B gene encodes a 594-amino-acid protein product that contains a Kelch domain in the C-terminal half (29). The Kelch domain is a common motif that forms a 4-stranded anti-parallel β-propeller. Kelch-repeat β-propellers interact with a variety of other proteins (30,31). Besides the presence of the Kelch domain and a verified expressed sequence tag (EST), no other information is available to determine the function of KLHDC7B (22). Kelch motif-containing proteins are involved in diverse biological processes, such as signal transduction, building cell structures, regulating transcription, metabolism and, notably, in stress responses (22,32,33). Mutations of Kelch proteins have been associated with cancer: Examples include KLHL6 in lymphocytic leukaemia, KEAP1 in pulmonary papillary adenocarcinoma, KLHL20 in prostate cancer, and KLHL37 (ENC1) in brain tumours (34). A recent study showed that apoptosis of MCF-7 decreased and proliferation increased when KLHDC7B was upregulated, and when KLHDC7B was downregulated, the opposite occurred, indicating its oncogenic properties (19). However, the encoded protein and its role in these cancers remain largely unknown.

Materials and methods

Tumour samples. Breast cancer specimens (n=26) and adjacent healthy tissue specimens (n=17) were obtained from female patients with breast cancer at Vall d'Hebron Hospital (Barcelona, Spain). The study was approved by the Clinical Research Ethics Committee at Vall d'Hebron Hospital [PR(AG)309/2016], and written informed consent was obtained from patients prior to sample collection. Tissues were extracted during 2009 and mRNA was extracted from 2015 to 2017. The selection criteria allowed different tumour types (papillary, ductal, lobular, mucinous, tubular and ductal) and grades, including metastatic and non-metastatic tumours.

Tumours were classified according to the Elston-Ellis modification of the Scarff-Bloom-Richardson (SBR) grading system (Nottingham grading system) (6) as well differentiated (grade 1, n=5), moderately differentiated (grade 2, n=10) or poorly differentiated (grade 3, n=11).

Histology and immunohistochemistry. Immunohistochemical staining were performed on five-micron-thick sections from formalin fixed and paraffin embedded (FFPE) tissues, on the Ventana Benchmark XT Automated IHC Stainer, using the Ventana ultraView Universal DAB Detection kit (760-500). After deparaffinization with Ventana EZ Prep solution (950-102), antigen retrieval was performed using Ventana Tris-based buffer solution CC1 pH 8 (950-124). Endogenous peroxidase was blocked with 3% hydrogen peroxide. After rinsing using Reaction Buffer (950-300), slides were incubated at 37˚C with each primary antibody (Ventana Medical Systems Inc, Tucson, AZ, USA; EEUU): Ki67-20 min (rabbit monoclonal antibody, 790-4286), p53-44 min (mouse monoclonal, 800-2912), HER2/neu-28 min (rabbit monoclonal, 790-2991), ER-40 min (rabbit monoclonal, 790-4324) and PR-16 min (rabbit monoclonal, 790-2223). Following incubation with HRP Multimer secondary antibody, primary antibodies-horseradish peroxidase-labelled antibody complex were visualized using diaminobenzidine tetrahydrochloride chromogen. Slides were then counterstained for 8 min with haematoxylin (760-2021), for 4 min with bluing reagent (760-2037), dehydrated and mounted. Appropriate positive and negative controls were included within the study sections.

Cell culture and reagents. MCF-10A, MCF-7, MDA-MB-231 and MDA-MB-468 breast cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and authenticated by DNA profiling using short tandem repeat (STR) (GenePrint® 10 System, Promega, Fitchburg, WI, USA) at Genomics Core Facility, Instituto de Investigaciones Biomédicas 'Alberto Sols' CSIC-UAM (Madrid, Spain) (35). Mycoplasma PCR analysis detected no genetic material. Cells were maintained at 37˚C in a 5% CO_2 humidified incubator (AutoFlow UN-5510, Nuaire, Plymouth, MN, USA). MCF-7, MDA-MB-231 and MDA-MB-468 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biowest, Nuaillé, France) and antibiotics (penicillin, streptomycin; Gibco-ThermoFisher Scientific, Waltham, MA, USA). MCF-10A medium was additionally supplemented with 20 ng/ml EGF (cat. no: E9644; Sigma, St. Louis, MO, USA), 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin (cat. no: C9903; Sigma) and 10 μg/ml insulin (cat. no: I9278; Sigma). Cells were trypsinized and passaged using TrypLE reagent (ThermoFisher Scientific).

Table I. Cell line characterization: Oestrogen receptor, progesterone receptor and p53 status, tumour subtype, origin and morphology.

Cell line	ER	PR	HER2	p53	Subtype	Origin	Morphology
MCF-10A	-	-	-	Wild type	Non-tumour	Fibrocystic disease	Epithelial
MCF-7	+	+	-	Wild type	Luminal A	Adenocarcinoma	Most differentiated; tight cell-cell junctions
MDA-MB-468	-	-	-	Mutated	Triple negative A	Adenocarcinoma	Core basal-like
MDA-MB-231	-	-	-	Mutated	Triple negative B	Adenocarcinoma	Least differentiated and highly invasive

ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

MCF-10A is a non-tumour breast cell line, which is hormone-receptor [oestrogen-receptor (ER) and progesterone-receptor (PR)] negative, HER2 negative and p53 wildtype (36-38) (Table I). The three breast cancer cell lines derive from breast adenocarcinomas (Table I). MCF-7 is classified as luminal A molecular subtype, hormone-receptor positive, HER2 negative and p53 wildtype. Luminal A cancers are low-grade, tend to grow slowly and have the best prognosis. MDA-MB-231 and MDA-MB-468 are triple-negative/basal-like breast cancer cell lines, hormone-receptor negative, HER2 negative and p53 mutated. Although both the MDA-MB cell lines are triple-negative, they show significant differences: MDA-MB-468 is classified as type A, showing a core basal-like morphology, and MDA-MB-231 is classified as type B, being the least differentiated, highly invasive and having the worst prognosis (39,40).

RNA extraction and quantification. Tissue samples were lysed using Tissue Lyser II (Qiagen, Venlo, the Netherlands) and RNA was extracted by the L'Hospital Universitari Vall d'Hebron Biobank (HUVH Biobank, Barcelona, Spain), using QuickGene RNA tissue SII kit (RT-S2) (Fujifilm, Neuss, Germany) in the automated nucleic acid extraction system QuickGene 810 (Fujifilm), according to the manufacturer's instructions. RNA from culture cell lines was extracted using the PureLink™ RNA Mini Kit (ThermoFisher Scientific). Quantification and assessment of RNA purity was performed using a NanoDrop ND2000 Spectrophotometer (ThermoFisher Scientific) and confirmed according to the RIN (RNA integrity number) using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

RT-PCR. One microgram of total RNA was used to synthesize cDNA using Maxima Reverse Transcriptase (ThermoFisher Scientific) on a Veriti 96-well Thermal Cycler (Applied Biosystems, ThermoFisher Scientific). RT-qPCR was performed according to the manufacturer's instructions, on an Applied Biosystems ABI 7500 Fast Real Time-PCR sequence detection system, using Taqman Technology (ThermoFisher Scientific): TaqMan GeX Master Mix (4369016), KLHDC7B probe (Hs00536653_s1) and HPOL probe (Hs00172187_m1) as housekeeping. RNA from healthy tissue was used as a normalisation control: FirstChoice® Human Breast Total RNA (AM6952, AppliedBiosystems-Ambion-Thermo Fisher). Analysis of relative gene expression data was conducted using RT-qPCR and the $2^{-\Delta\Delta Cq}$ method (41).

The average KLHDC7B expression for grade 1 (G1) tumours (n=5) was calculated, and mRNA expression of every tumour was reported as relative to this average (value=1). The two G1 tumours with the highest KLHDC7B expression were used as cut-offs for low expression (G1 tumours being those with the lowest KLHDC7B expression overall) (Table II).

Comparison between healthy and tumour tissue was performed in cases in which there was enough mRNA from healthy tissue to perform retrotranscription from mRNA to cDNA.

Protein extraction and immunoblotting. Total protein extracts were generated using a RIPA Lysis Buffer System (sc24948; SantaCruz Biotechnology, Dallas, TX, USA) supplemented with Protease Inhibitor Cocktail set III (539134; Calbiochem, San Diego, CA, USA) and Phosphatase Inhibitor Cocktail Set II (524625, Calbiochem). Protein was quantified using a BCA Protein Assay kit (23225; ThermoFisher Scientific). Protein extracts (15 μg per sample) were loaded onto SDS-PAGE gels and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% BSA in TBS-T (Tris-buffered saline, 0.1% Tween 20). Membranes were incubated with KLHDC7B antibody (ab126063; Abcam, Cambridge, UK) diluted at a ratio of 1:500 according to the manufacturer's instructions and incubated with the membranes overnight at 4˚C. Goat horseradish peroxidase (HRP)-linked secondary antibody was added at a dilution ratio of (1:5,000) (31460; Pierce ThermoScientific) and incubated with the membranes at room temperature for 1 h. β-actin (JLA20; Calbiochem) was used as housekeeping (1:15,000, 1 h at room temperature, secondary antibody not required). The membranes were washed 3 times with TBS-T. Immunodetection of proteins was performed using Amersham ECL Western Blotting Detection Reagent (GE Healthcare, Chicago, IL, USA) according to the manufacturer's instructions.

Statistical analysis. Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA) was used for statistics and data representation. Data are presented as the mean ± standard error of the mean. Significant differences were determined using ANOVA with Dunnett's multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Table II. Tumour features.

Patient ID	Tumour type (breast carcinoma)	Grade	ER	PR	HER	p53	Ki67 (% positive cells)	Vascular invasion	Metastases	Relative mRNA expression (to G1 average=1)
B09-13526	Papillary	2	+++	+++	-	-	5-10	-	No	0.18
B09-3889	Ductal	2	?	?	?	?	?	-	No	0.43
B09-24083	Lobular	1	+++	+++	-	-	<5	-	Yes	0.58
B09-16306	Mucinous	1	+++	+++	-	-	<1	-	No	0.60
B09-12616	Cribriform/tubular	1	+++	+++	-	?	?	-	No	0.77
B09-5308	Ductal	3	?	?	?	?	?	-	No	0.77
B09-18339	Lobular	2	+++	+	-	-	15	-	No	0.87
B09-11839	Ductal	2	+++	-	-	-	5	-	Yes	0.91
B09-18841	Ductal	3	+++	+++	-	-	5	-	Yes	1.03
B09-16005	Ductal	3	+++	+++	-	+	60	+	Yes	1.09
B09-988	Lobular	2	?	?	?	?	?	?	Yes	1.21
B09-25265	Lobular	2	+++	+++	-	-	2	-	Yes	1.21
B09-19643	Lobular	2	?	?	?	?	?	?	No	1.34
B09-24926[a]	Ductal	1	+++	+++	-	-	<5	+	Yes	1.48
B09-24052	Ductal	3	+++	+++	-	-	20	-	No	1.52
B09-22493	Lobular	2	+++	+++	-	-	<5	-	No	1.63
B09-24372[a]	Ductal	1	+++	+++	-	-	1	-	No	1.64
B09-8511	Ductal	2	+++	+++	-	-	15	-	Yes	1.69
B09-7717	Ductal	3	+++	++	-	+	70	-	?	1.79
B09-11451	Papillary	3	+++	++	-	?	?	-	?	1.80
B09-3173	Ductal	3	-	-	-	+	>70	+	Yes	1.85
B09-26055	Ductal	3	+++	+	-	-	40	-	Yes	1.98
B09-1573	Tubular	2	?	?	?	?	?	?	Yes	2.11
B09-20004	Ductal	2	+++	+++	-	-	3	-	No	2.16
B09-15644	Ductal	2	+++	++	-	-	15-20	+/-	Yes	2.26
B09-20128	Papillary/Ductal	3	+++	+++	-	-	10	-	No	2.43
B09-20045	Ductal	3	-	-	-	+	60	-	No	3.02
B09-25449	Ductal	2	++	+++	-	-	25	+	Yes	3.70
B09-2726	Ductal	3	-	-	-	+	50	+	Yes	3.98
B09-19151	Ductal	3	+++	+++	-	+	1	-	No	6.98
B09-24264	Ductal	2	+++	+++	-	-	<5	-	No	23.44
B09-17267	Ductal	3	++	+	+	+	15-20	+	No	272.58

[a]The two G1 tumours with the highest KLHDC7B expression, used as the cut-off for low expression. Average expression in G1 tumours (n=5) was calculated, and mRNA expression in each tumour was reported as relative to this average (value=1). ER, oestrogen receptor; PR, progesterone receptor; HER, human epidermal growth factor receptor; ?, unknown.

Results

Clinicopathological evaluation and KLHDC7B characterization in breast cancer tumours. We studied the expression of KLHDC7B mRNA in tumours from different pathological grades (Fig. 1A). Our results showed that KLHDC7B expression tended to increase as tumour grade increased from grade 1 to 2. Grade 3 tumours showed a significant upregulation of KLHDC7B.

Table II shows the classification of tumours in order of increasing KLHDC7B expression, with information on tumour type, tumour grade, hormone-receptor (oestrogen and progesterone), HER and p53 status, percentage of Ki67 positive cells, metastasis and vascular invasion. Ki67 cells and tumour type showed a correlation with KLHDC7B expression. Tumours with more than 10% Ki67 cells had the highest levels of KLHDC7B expression. For tumour type, lobular tumours had the lowest expression of KLHDC7B (83.33% of lobular tumours were classified as having low expression), and ductal tumours had the highest expression (68% of ductal tumours were classified as having high expression). Papillary, mucinous, cribriform and tubular tumours were also analysed, but due to the low number of each of these tumour types, we cannot draw conclusions on their relationship to KLHDC7B expression. Besides tumour type and percentage of positive Ki67 cells, no other correlations were found between KLHDC7B and the tumour features described above.

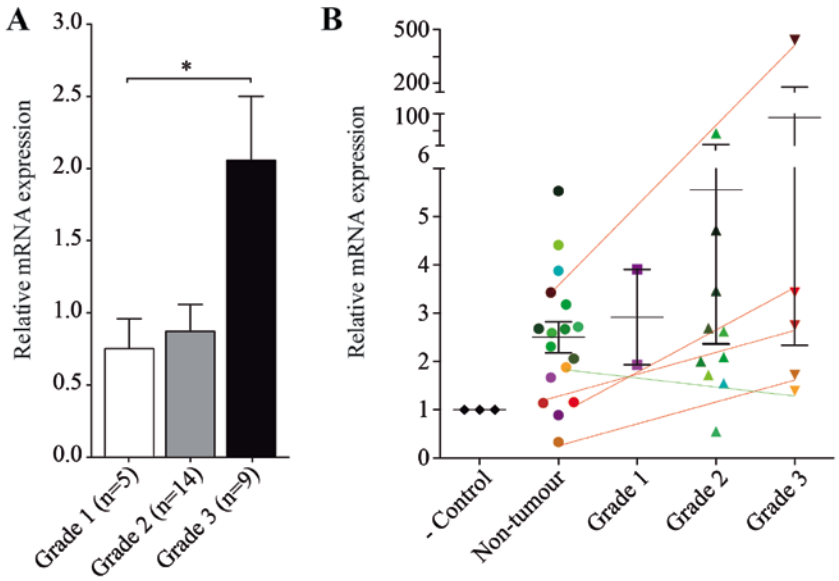

Figure 1. KLHDC7B mRNA expression in breast cancer. (A) mRNA expression in grade 1 (n=5), grade 2 (n=14) and grade 3 (n=9) breast tumours, relative to grade 1 tumours. (B) Relative mRNA expression in negative control (commercial RNA), non-tumour surrounding breast tissue, and grade 1, 2 and 3 tumours. Expression relative to negative control. For each tumour grade, there is a corresponding mark of the same colour in non-tumour tissue, allowing a comparison. Lines connect grade 3 tumours with their respective non-tumour surrounding tissue (red, upregulated expression; green, downregulated expression). *P<0.05, as indicated. KLHDC7B, Kelch domain-containing protein 7B.

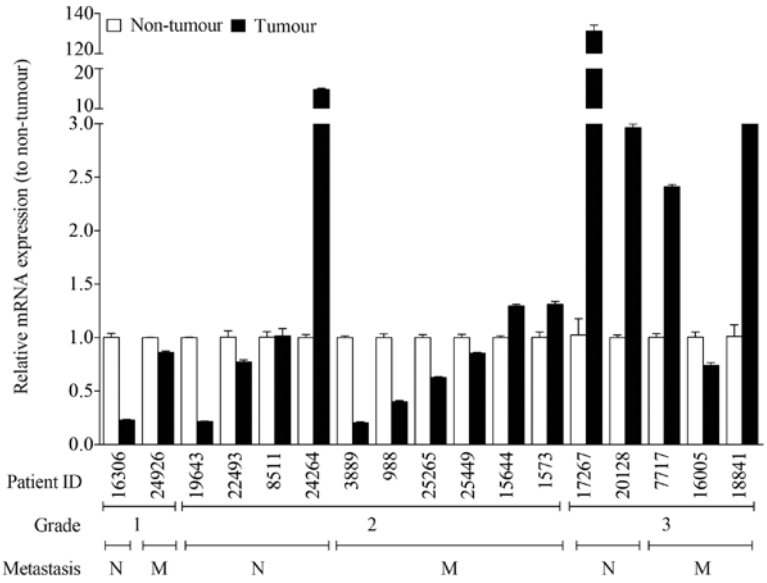

Figure 2. mRNA expression in breast tumours compared with non-tumour surrounding tissue in the same patient. Expression relative to non-tumour tissue. The size of the error bars reflects the variation in the replicates. KLHDC7B, Kelch domain-containing protein 7B; Metastasis: N, No metastasis; M, Metastasis.

In comparison to healthy tissue from the area surrounding the tumour, with expression normalised to a commercial RNA sample from a healthy donor (Fig. 1B), grade 3 tumours showed a tendency to KLHDC7B upregulation. However, the expression of KLHDC7B in grade 3 tumours was not always higher than non-tumour samples from other patients (from grade 1- and 2-matched tissue).

To avoid the influence of tumour heterogeneity among patients, we compared the KLHDC7B expression in breast tumours and in the surrounding healthy tissue in the same patient (Fig. 2). Seven out of 12 (58%) grade 1 and 2 tumours showed a significantly lower KLHDC7B expression compared to non-tumour surrounding tissue. Only 3 out of 10 (30%) grade 2 tumours showed a significantly higher KLHDC7B expression in tumour tissue. Four out of 5 (80%) grade 3 tumours showed a significantly higher KLHDC7B expression than healthy tissue.

When we correlated KLHDC7B expression with metastatic capacity (Fig. 2), 4 out of 10 (40%) of the tumours that produced metastases showed upregulation of this gene, but 5 out of 10 (50%) had downregulation.

Characterization of KLHDC7B mRNA and protein expression in cell lines. KLHDC7B mRNA expression was evaluated in

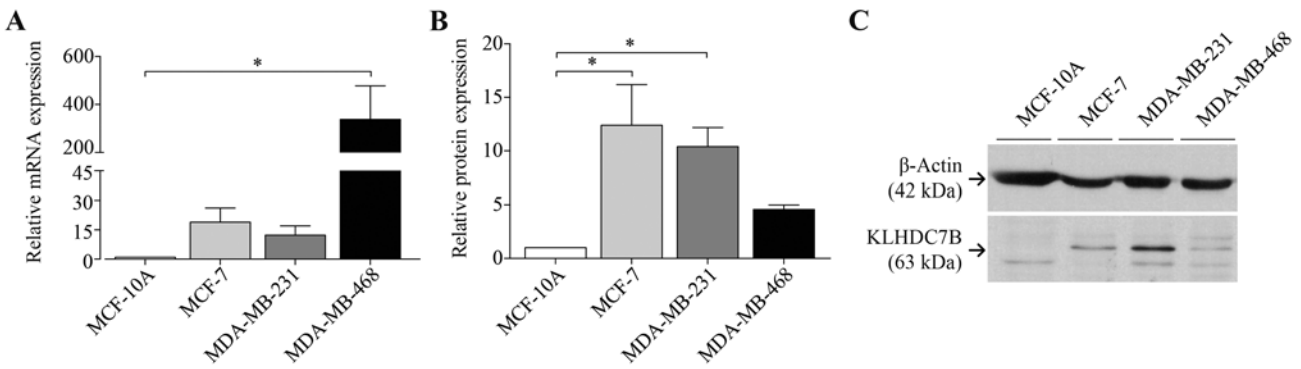

Figure 3. KLHDC7B expression in breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-468) compared with the non-tumour cell line (MCF-10A). (A) mRNA expression, relative to MCF-10A. (B) Protein expression via western blotting, relative to MCF-10A. (C) Representative image of protein expression as determined by western blotting. β-actin was used as the internal control. *P<0.05, as indicated. KLHDC7B, Kelch domain-containing protein 7B.

different cell lines, using MCF-10A as the non-tumour cell lines and MCF-7, MDA-MB-231 and MDA-MB-468 as the tumour cell lines. As expected, KLHDC7B was overexpressed in tumour cell lines (Fig. 3A).

Protein expression was determined by western-blot (Fig. 3B and C). All four tumour cell lines showed higher expression than MCF-10A (no expression). Surprisingly, MDA-MB-468 showed lower expression than the other tumour cell lines, although the mRNA expression was higher.

Discussion

KLHDC7B has been found in different tumours including breast, ovary and cervical cancer (13,17-18,20), revealing its possible role in tumour biology. The KLHDC7B gene has been found to be hypermethylated and upregulated in breast cancer and consequently has been postulated as an epigenetic marker of breast cancer (17). However, its role in the development and progression of these cancers is largely unknown.

To understand the role of KLHDC7B in tumour progression we studied the expression of KLHDC7B mRNA in tumours of different pathological grades. Grade 3 tumours, tumours with more than 10% Ki67 positive cells and ductal tumours had the highest expression of KLHDC7B (Table II). Ki67 expression is a well-known marker of active proliferation and the association between high proliferation and poor prognosis is well stablished (42-44). Lobular, mucinous, tubular, and papillary carcinomas have been associated with lower risk of mortality than ductal carcinomas (45). Together, these data indicate that KLHDC7B is associated with more aggressive tumours and worse prognosis.

The upregulation of KLHDC7B in advanced tumours could suggest a positive association with metastatic capacity, although we did not find such a difference in our analysis.

When we compared tumour tissue with healthy breast tissue, KLHDC7B expression in tumour tissue was not always higher than in non-tumour samples from other patients with grade 1 and 2 tumours. These data reveal a huge variability among individuals, demonstrating one of the most relevant issues in oncology-intertumour heterogeneity. To improve understanding of the role of KLHDC7B in breast cancer, we compared KLHDC7B expression in breast tumours and surrounding healthy tissue from the same patient. This new approach confirmed previously published data on KLHDC7B upregulation in breast tumours (17), but our results also revealed that the expression of this gene is grade-dependent and only significantly upregulated in grade 3 tumours. Additionally, we found interesting results in grade 1 and 2 tumours, that KLHDC7B was downregulated in well-differentiated and moderately-differentiated tumours. These new data would suggest a dual role of KLHDC7B during tumour progression, which we will analyse in future studies.

We can conclude that when using KLHDC7B expression as a marker of breast cancer, it should be correlated against healthy tissue from the same patient, rather than the general population, as comparisons with the general population are likely to lead to false results (a consequence of intertumour heterogeneity). Additionally, use of KLHDC7B as a marker without considering tumour grade could lead to inaccurate diagnoses.

The results of this study could increase understanding of the involvement of KLHDC7B in breast cancer, although the sample size poses a potential limitation, and future studies should use a larger sample.

These data indicate that KLHDC7B is associated with more aggressive tumours and worse prognosis, however they do not explain the functional role of KLHDC7B in breast tumours. KLHDC7B protein could have an anti- or pro-tumour role or even a dual role that could explain the differences according to tumour grade. To unravel the functional role of KLHDC7B, future experiments should be performed including up- and down-regulation of KLHDC7B expression to establish its role in the progression of breast tumours. These studies should preferably be performed in breast cell lines.

We studied the expression of KLHDC7B in tumour cell lines and healthy cell lines. MCF-10A, MDA-MB-231 and MDA-MB-468 are hormone-receptor and HER negative cell lines; MCF-10A does not express KLHDC7B, while the two MDA-MB cell lines do. In contrast, MCF-7 is a hormone-receptor positive cell line and expresses KLHDC7B (Table I). Our results therefore suggest that hormone-receptor and HER status are not related to KLHDC7B expression.

We have confirmed previously published results on mRNA expression and added protein expression studies, which have not previously been described. Our data show that mRNA expression does not correlate exactly with protein expression. A previous study by Gry *et al* (46) showed that RNA does

not always correlate with protein expression and, more importantly, the correlation of the same protein can vary depending on the cell line. This weak correlation is due to several factors, including various post-transcriptional processes: For example, some mRNAs are strongly retained in the nucleus, which may lead to overestimation of RNA levels relative to protein levels. The lack of correlation between RNA and protein levels in MDA-MB-468 cells could be the result of complex regulatory mechanisms. For future *in vitro* studies, protein expression should be taken into consideration when selecting the appropriate cell line model, and we recommend that MDA-MB-468 not be used as a model to study the role of KLHDC7B protein in breast cancer.

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Availability of data and materials

All data generated or analysed during the present study are included in this published article.

Authors' contributions

AMP designed and carried out the experiments, interpreted the results, and wrote the manuscript. SRYC obtained the human samples and patient data, and contributed to project funding and manuscript revision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The project was approved by the Clinical Research Ethics Committee at Vall d'Hebron Hospital (PR(AG)309/2016). Patients provided written informed consent for sample collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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