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Identifying microRNAs regulating B7-H3 in breast cancer: the clinical impact of microRNA-29c

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Background: B7-H3, an immunoregulatory protein, is overexpressed in several cancers and is often associated with metastasis and poor prognosis. Here, our aim was to identify microRNAs (miRNAs) regulating B7-H3 and assess their potential prognostic implications in breast cancer.

Methods: MicroRNAs targeting B7-H3 were identified by transfecting two breast cancer cell lines with a library of 810 miRNA mimics and quantifying changes of B7-H3 protein levels using protein lysate microarrays. For validations we used western immunoblotting and 3′-UTR luciferase assays. Clinical significance of the miRNAs was assayed by analysing whether their expression levels correlated with outcome in two cohorts of breast cancer patients (142 and 81 patients).

Results: We identified nearly 50 miRNAs that downregulated B7-H3 protein levels. Western immunoblotting validated the impact of the 20 most effective miRNAs. Thirteen miRNAs (miR-214, miR-363*, miR-326, miR-940, miR-29c, miR-665, miR-34b*, miR-708, miR-601, miR-124a, miR-380-5p, miR-885-3p, and miR-593) targeted B7-H3 directly by binding to its 3′-UTR region. Finally, high expression of miR-29c was associated with a significant reduced risk of dying from breast cancer in both cohorts.

Conclusions: We identified miRNAs efficiently downregulating B7-H3 expression. The expression of miR-29c correlated with survival in breast cancer patients, suggesting a tumour suppressive role for this miRNA.

Breast cancer is the most common cancer in women worldwide, with about 1.38 million new cases and 458 000 deaths each year (Ferlay *et al*, 2010). The majority of breast cancer morbidity and mortality is due to incurable metastatic disease that is highly resistant to conventional therapies. To reduce breast cancer mortality it is therefore essential to further elucidate the molecular mechanisms of breast cancer metastasis, and develop novel therapeutic approaches. Although decades of metastasis research

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have provided considerable insight into the multistep metastatic process, there are still significant gaps in our knowledge.

Recently, it has been recognised that microRNAs (miRNAs) affect various steps in breast cancer metastasis including epithelial-to-mesenchymal and mesenchymal-to-epithelial transition (EMT–MET transition), migration, invasion and angiogenesis and could represent potential therapeutic targets (Harquail *et al*, 2012; Wang *et al*, 2012). MicroRNAs are short non-coding RNAs that by targeting messenger RNAs (mRNAs) are key post-transcriptional regulators of gene expression (Ambros, 2001; Lai, 2002). By binding to the 3′-untranslated region (3′-UTR) of their target mRNA, miRNAs inhibit translation and/or promote mRNA degradation (Valencia-Sanchez *et al*, 2006). Consequently, they can act either as oncogenes or as tumour suppressors depending on their target mRNAs (Garzon *et al*, 2009; Shenouda and Alahari, 2009). The binding of an miRNA to an mRNA is not exclusive, as a single miRNA can target hundreds of mRNAs, and a single mRNA can be affected by multiple miRNAs (Selbach *et al*, 2008).

B7-H3, an immunoregulatory protein that belongs to the B7 family of T-cell co-regulatory molecules (Collins *et al*, 2005) is overexpressed in several different cancer forms and often associated with metastasis and poor prognosis (Sun *et al*, 2006; Roth *et al*, 2007; Crispen *et al*, 2008; Yamato *et al*, 2009; Zang *et al*, 2010; Katayama *et al*, 2011; Ingebrigtsen *et al*, 2012; Sun *et al*, 2012). In breast cancer, tumour B7-H3 expression is significantly correlated with more advanced disease and lymph node metastasis (Arigami *et al*, 2010; Liu *et al*, 2013). Whereas most reports emphasise the immunoregulatory function of B7-H3, we have demonstrated a non-immunological role of B7-H3 in cancer progression and metastasis, also supported by the work of Yuan *et al* (2011).

On the basis of the emerging evidence for miRNAs as regulators of cancer metastasis and our interest in B7-H3 as a metastasis-associated protein, we wanted to identify B7-H3-targeting miRNAs and to assess whether these have prognostic implications in breast cancer. It has previously been shown that miR-29 directly targets B7-H3 and leads to downregulation of the B7-H3 protein level in neuroblastoma, sarcoma and brain tumours (Xu *et al*, 2009) as well as in melanoma (Wang *et al*, 2013). In the present study, we evaluated the effects of 810 miRNAs on B7-H3 and phospho (p)-Stat3 protein levels, by utilising a protein lysate microarray (LMA) technology (Leivonen *et al*, 2009). We identified nearly 50 miRNAs downregulating B7-H3 and validated the 20 most effective miRNAs. We demonstrated that 13 of the 20 selected miRNAs, including miR-29c, targeted B7-H3 directly by binding to its 3′-UTR region. Finally, we showed that high expression of miR-29c is associated with increased survival in two cohorts of breast cancer patients.

MATERIALS AND METHODS

Cell culture and reagents. JIMT-1 cell line was procured from DSMZ (Brauenschweig, Germany) and grown in 1 : 1 DMEM/F12 (both from Mediatech, Inc., Manassas, VA, USA) media supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals Inc., Lawrenceville, GA, USA), 1% glutaMAX (Gibco, Paisly, UK), 10 $\mu g\,ml^{-1}$ insulin (Sigma Chemicals, Perth, WA, Australia) and 1% penicillin–streptomycin (Lonza BioWhittaker, Walkersville, MD, USA). The KPL-4 cell line was a kind gift from Professor Junichi Kurebayashi (Kawasaki Medical School, Japan) and was grown in DMEM supplemented with 10% FBS, 1% glutaMAX and 1% penicillin–streptomycin. Both cell lines were kept at 37 °C in 5% CO_2 and routinely tested for mycoplasma infections using the ATCC Mycoplasma detection kit (ATCC, Manassas, VA, USA).

Twenty different human mirVANA miRNA Mimics (hsa-miR-892a, hsa-miR-380-5p, hsa-miR-125b-2*, hsa-miR-363*, hsa-miR-940, hsa-miR-214, hsa-miR-34b*, hsa-miR-665, hsa-miR-593, hsa-miR-29c, hsa-miR-555, hsa-miR-885-3p, hsa-miR-567, hsa-miR-297, hsa-miR-187-3p, hsa-miR-124a-1, hsa-miR-326, hsa-miR-601, hsa-miR-506 and hsa-miR-708) and the mirVANA miRNA mimic negative control #1 were purchased from Life Technologies (Grand Island, NY, USA).

LMA screening and data analysis. The LMA screening has been described in detail previously (Leivonen *et al*, 2009, 2014). Briefly, JIMT-1 and KPL-4 were transfected with a human miRIDIAN microRNA mimic library v.10.1 (altogether 810 miRNA mimics) (Dharmacon, Lafayette, CO, USA) at a 20-nM concentration in 384 wells using siLentFect (Bio-Rad Laboratories, Hercules, CA, USA). After 48 or 72 h of incubation, cells were lysed and printed on nitrocellulose-coated microarray FAST slides (Whatman Inc., Florham Park, NJ, USA). B7-H3 and p-Stat3 protein expression were detected by staining the slides with a B7-H3 antibody (#AF1027) and p-Stat3 antibody (#9131) (both purchased from R&D, Minneapolis, MN, USA), followed by exposure to Alexa Fluor 680-tagged secondary antibodies (Invitrogen Inc., Carlsbad, CA, USA). For total protein measurement, arrays were stained with Sypro Ruby Blot solution (Invitrogen). The slides were scanned with a Tecan LS400 (Tecan Inc., Durham, NC, USA) microarray scanner and an Odyssey Licor IR-scanner (LI-COR Biosciences, Lincoln, NE, USA) to detect Sypro, B7-H3 and p-Stat3 signals, respectively. Array-Pro Analyzer microarray analysis software (Median Cybernetics Inc., Bethesda, MD, USA) was used for analysing data. The B7-H3 and p-Stat3 data were normalised to the Sypro total protein, $\log_2$ transformed and z-score standardized. z-scores < -2.0 were considered as a significant downregulation.

Transfection, SDS–PAGE and immunoblotting. To validate the LMA data, the cells were transfected with 20 nM mirVANA miRNA Mimics or 20 nM mirVANA miRNA mimic negative control #1 using siLentFect according to the manufacturer's protocol. After 72 h the cells were lysed in 1 × SDS sample buffer (NuPage, Life Technologies, Carlsbad, CA, USA) supplemented with reducing agent (NuPage, Life Technologies). The samples were heated at 95 °C for 10 min and then separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in 4–12% gels (Life Technologies) and transferred onto PVDF membranes (Bio-Rad Laboratories). Blotted membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (TBST) and 5% non-fat dry milk for 1 h at room temperature and probed overnight at 4 °C with specific monoclonal or polyclonal antibodies (B7-H3, phospho-Stat3 or total Stat3) in TBST with 5% non-fat dry milk or bovine serum albumin. Anti-β-actin (#sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control. The horseradish peroxidase conjugated secondary antibodies were all from Dako (Glostrup, Denmark). Signals were developed with Super Signal West Dura Extended Duration Substrate (Pierce, Thermo Scientific, Rockford, IL, USA) and measured by the digital image station (Kodak, Rochester, NY, USA).

3′-UTR reporter constructs and luciferase assays. The B7-H3 3′-UTR GoClone contruct was purchased from SwitchGear Genomics (MenloPark, CA, USA). An empty 3′-UTR GoClone (SwitchGear) was used as a positive control. For luciferase assays, JIMT-1 cells (10 000 per well) were plated onto 96-well plates 24 h before transfection. The cells were co-transfected with 100 ng of B7-H3 3′-UTR GoClone construct and 50 nM mirVANA miRNA Mimic using Lipofectamine 2000 (Life Technologies) as a transfection reagent according to the manufacturer's protocol. Luciferase activity was assayed 24 h after transfection with a Dual-Glo Luciferase Assay System (Promega Corp., Madison, WI, USA) and

was measured with a Wallac Victor2 plate reader (Perkin Elmer, Waltham, MA, USA).

Patient material. The DBCGbc patient material included 142 tumours from high-risk Danish breast cancer patients from the DBCG82bc cohort, with >20 years of follow-up information. Premenopausal women (DBCGb protocol) were randomised to receive radiation therapy and CMF (cyclophosphamide, methotrexate, fluorouracil; eight cycles) or only CMF chemotherapy (nine cycles). Postmenopausal women (DBCGc protocol) were randomised to receive radiation therapy + tamoxifen (30 mg daily for 1 year) or tamoxifen alone (Kyndi *et al*, 2008). Oral informed consent was mandatory, and the study of the DBCG82bc cohort was approved by the Regional Ethical Committee (Journal number 20030263). Gene expression, miRNA expression, ER status, HER2 status and *TP53* mutation status were used for analysis on this data set (Kyndi *et al*, 2008; Myhre *et al*, 2010; Aure *et al*, 2013).

The MicMa patient material consisted of 101 stage I and II breast cancer patients diagnosed in 1995–1998 with a median time of follow-up of 85 months. These were part of a larger patient material consisting of 920 patients with clinical information of disseminated tumour cell status (Wiedswang *et al*, 2003). The study was approved by the regional ethical committee (S-97103), and all patients have given informed written consent. Gene expression, miRNA expression, ER status, HER2 status and *TP53* mutation status have been described before (Enerly *et al*, 2011). For 81 samples, full clinical information was available and those were used for the survival analyses.

MicroRNA expression analyses. For MicMa, the tumour RNA from fresh frozen samples was isolated using TRIzol reagent (Invitrogen) (Enerly *et al*, 2011), and for DBCGbc with the Qiagen Midi kit Extraction column procedure (Qiagen GmbH, Hilden, Germany) (Aure *et al*, 2013). The miRNA expression profiling for both cohorts was performed with the 8 × 15 k 'Human miRNA Microarray Kit (V2)' (design id 019118) from Agilent (Agilent Technologies, Santa Clara, CA, USA). Briefly, 100 ng total RNA was dephosphorylated, labelled and hybridised for 20 h following the manufacturer's protocol. Scanning was performed on Agilent Scanner G2565A, signals were extracted using Feature Extraction v.9.5 and the subsequent data processing was performed using the GeneSpring software v.12.0 (Agilent Technologies). For normalisation, the expression values were $\log_2$ transformed and normalised to the 90th percentile. MicroRNAs that were detected in <10% of the samples were excluded. The miRNA data have been previously published in the Gene Expression Omnibus (GEO) with accession number GSE19536 for the MicMa data (Enerly *et al*, 2011), and GSE46934 for the DBCGbc data (Aure *et al*, 2013).

Quantitative RT–PCR analyses. Total RNAs from JIMT-1 and KPL-4 cells were isolated with the MiRVANA kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Absolute quantification of B7-H3 levels was performed using the TaqMan Gene Expression assay Hs00987207_m1 (Applied Biosystems, Foster City, CA, USA). Beta-actin was used as an endogenous control (assay 4352935E). Absolute quantification of miR-29c was performed using the TaqMan MicroRNA assay 000587 (Applied Biosystems) with RNU6B as an endogenous control (assay 001093). The standard curves were set up as a two-fold dilution series in triplicates of known concentrations of FirstChoice Human Breast Total RNA (Applied Biosystems). RT–PCR reactions were carried out using the manufacturer's recommendation. In brief, 5 ng of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems) for B7-H3 and β-actin, and the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) with miRNA-specific RT-primers (Applied Biosystems) for miR-29c and RNU6B. Quantitative real-time PCR was performed following the manufacturer's recommendation in triplicates on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) with a standard absolute quantification thermal cycling program. Cycle threshold (C_t) values and absolute quantification were obtained using the SDS 2.3 software (Applied Biosystems).

Statistical analysis. The *in vitro* experiments were performed a minimum of four times, and all data are shown as mean ± s.e.m. The data were analysed for statistical significance using an unpaired Student's *t*-test. Univariate survival analysis was performed according to the Kaplan–Meier method, and survival was compared using the log-rank test. Statistical significance of miR-29c expression between molecular breast cancer subtypes and histologic grade was determined by the Kruskal–Wallis test. Correlation between miR-29c expression and B7-H3 mRNA expression was calculated using the Spearman Rank method, and association of miR-29c expression with the B7-H3 status was determined by the Mann–Whitney test. The SPSS version 18 software (SPSS Inc., Chicago, IL, USA) and STATA (STATA Corp., College Station, TX, USA) were used for statistical calculations. For all the experiments, any *P*-value below 0.05 was considered statistically significant.

RESULTS

Protein LMA to identify miRNAs regulating B7-H3 in breast cancer cell lines. We applied protein LMA screening (Leivonen *et al*, 2009) to identify B7-H3-regulating miRNAs in two breast cancer cell lines, KPL-4 and JIMT-1, which express B7-H3 at relatively high levels. The cells were transfected with a miRNA mimic library containing 810 miRNAs. After 48 and 72 h of incubation, the cells were lysed, printed on nitrocellulose-coated slides and stained with a specific antibody against B7-H3. As we previously have demonstrated that silencing B7-H3 affects the level of p-Stat3 (Liu *et al*, 2011), we also stained the slides with a specific p-Stat3 antibody. In addition, the slides were counterstained with Sypro Ruby to normalise the levels of B7-H3/p-Stat3 to total protein. The whole screening data with normalised *z*-scores are provided as a Supplementary Table S1.

In the JIMT-1 cell line, we initially identified 46 miRNAs that significantly reduced the protein expression of B7-H3 at a 48-hour time point (z-score < −2.0), compared with 48 at 72 h (Supplementary Table S1). For the KPL-4 cell line, 49 miRNAs significantly reduced the expression of B7-H3 after 48 h and 49 after 72 h (Supplementary Table S1). Interestingly, there were also miRNAs efficiently upregulating B7-H3 in both cell lines, for instance miR-518a-5p. However, as our aim was to find negative regulators of B7-H3, we did not select the upregulating miRNAs for further analyses.

We also identified 37 miRNAs that reduced p-Stat3 protein level after 48 h and 27 after 72 h (KPL-4, Supplementary Table S1). MiR-29c, which previously has been shown to regulate B7-H3 in neuroblastoma, sarcoma and brain cancers (Xu *et al*, 2009) was among the strongest hits in our screen.

Furthermore, we showed that there was a correlation between the *z*-score values after both 48 (Figure 1A) and 72 h (Figure 1B) for the JIMT-1 and the KPL-4 cell lines. Thus, 33 of 46 and 37 of 48 (48 and 72 h, respectively) (Supplementary Table S1, marked in bold) of the miRNAs effective in JIMT-1 cells were effective in KPL-4 cells as well. Conversely, 33 of 49 and 37 of 49 (48 and 72 h, respectively) (Supplementary Table S1, marked in bold) of the miRNAs with significant effects in KPL-4 cells were effective in JIMT-1 cells. Notably, a strong relationship between *z*-score values for B7-H3 and p-Stat3 expression after both 48 and 72 h was found (Supplementary Figure S1A and S1B).

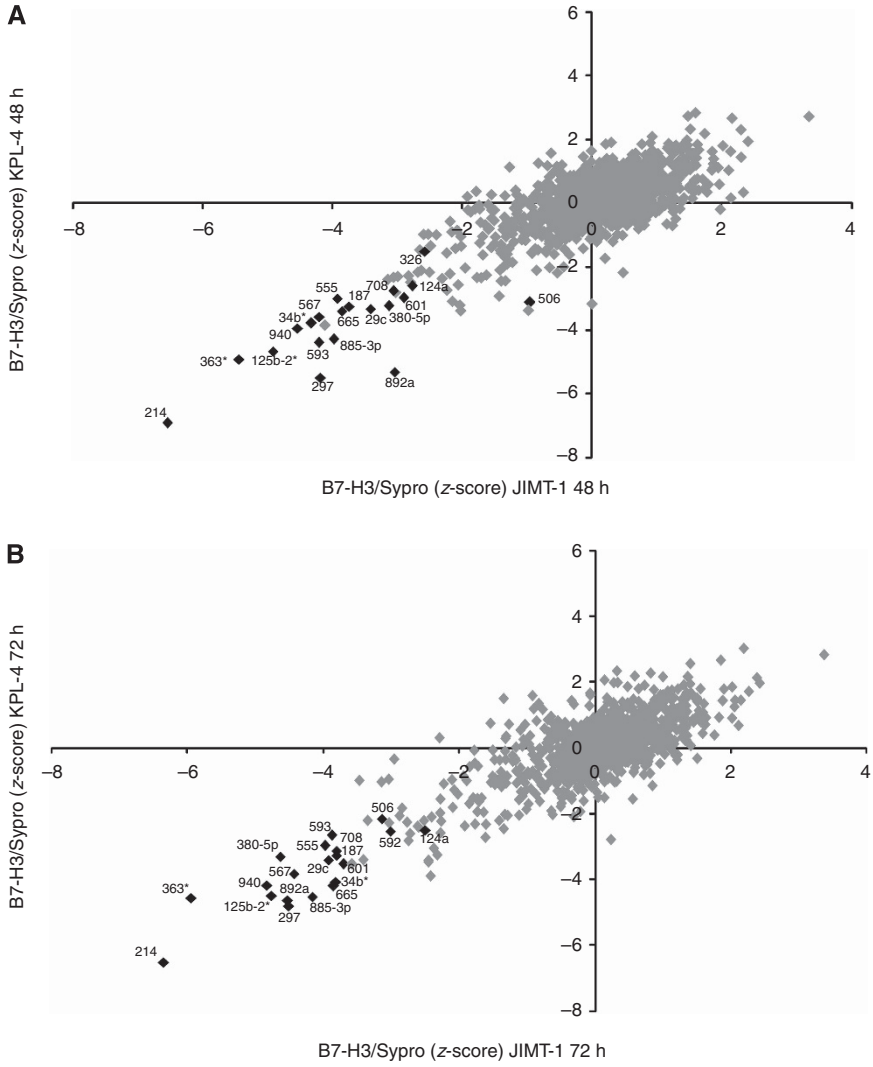

Figure 1. Protein lysate microarray (LMA) screen for identifying microRNAs (miRNAs) regulating B7-H3. (**A**) A scatter plot of a 48-h screen (JIMT-1 *vs* KPL-4). (**B**) A scatter plot of a 72-h screen (JIMT-1 *vs* KPL-4). Black spots indicate miRNAs that were selected for further validation by western immunoblotting and are marked with numbers representing miRNA names.

Validation of screening data by assessment of the B7-H3 and phosphorylated Stat3 protein levels. To validate the primary screening data, we selected the 17 most effective miRNAs downregulating B7-H3 (Table 1). In addition, we decided to validate miR-326, miR-505 and miR-124a based on their strong negative effect on the p-Stat3 levels in the KPL-4 cell line. Notably, we were not able to detect p-Stat3 in the JIMT-1 cell line in the screen.

The 20 selected miRNAs were transfected into the JIMT-1 and the KPL-4 cells, and after 72 h the cells were lysed and the protein levels of B7-H3 and p-Stat3 were analysed by western immunoblotting. In the JIMT-1 cells, we demonstrated that all the miRNAs downregulated B7-H3 protein expression as compared with negative control scrambled miRNAs (Figure 2A; Supplementary Figure S2). Consistent with the LMA results, p-Stat3 levels in JIMT-1 cells were low (results not shown). For the KPL-4 cell line, most of the miRNAs caused a reduction in the B7-H3 protein level (Figure 2B; Supplementary Figure S2). The level of downregulation varied among the different miRNAs, the miR-506 and miR-187-3p having the weakest influence on the B7-H3 level.

Finally, we investigated the phosphorylation status of Stat3 in the KPL-4 cell line and observed that 11 of the 20 miRNAs also reduced the level of p-Stat3. MiR-297, miR-34b*, miR-124a and miR-380-5p had a very weak or no influence on the p-Stat3 protein level although the B7-H3 protein level was reduced. This was in concordance with the LMA data and it might reflect that these miRNAs may exert their effects on B7-H3 via other target genes than those affecting Stat3.

Validating miRNAs binding directly to the B7-H3 3′-UTR. All the 20 miRNAs that were selected for validation were investigated by miRanda (Betel *et al*, 2008) and TargetScan algorithms (Grimson *et al*, 2007) for their potential interaction with B7-H3 3′-UTR. We found that 13 of the selected miRNAs were predicted to bind to B7-H3 3′-UTR (Table 2), which contains a sequence of ~1500 bases.

To examine whether the 20 miRNAs actually could bind to the B7-H3 3′-UTR, we took advantage of the LightSwitch miRNA Target Validation System (SwitchGear). We used a luciferase reporter construct (LightSwitch 3′-UTR reporter GoClone) with B7-H3 3′-UTR sequence using the novel RenSP luciferase technology (SwitchGear). The reporter was co-transfected into JIMT-1 cells with miRNAs and the luciferase activity was measured after 24 h. Transfection of miR-214, miR-363*, mir-326, miR-940, miR-29c, miR-665, miR-34b*, miR-708, miR-601, miR-124a, miR-380-5p, miR-885-3p and miR-593 significantly inhibited the

Table 1. B7-H3 downregulation (z-score) (JIMT-1 and KPL-4) combined with p-Stat3 downregulation (z-score) (KPL-4) for the miRNAs selected for validation

	B7-H3/sypro z-score		B7-H3/sypro z-score		p-Stat3/sypro z-score	
	JIMT-1		KPL-4		KPL-4	
miRNA	48 h	72 h	48 h	72 h	48 h	72 h
hsa-miR-214	−6.54	−6.35	−6.92	−6.53	−2.16	−2.28
hsa-miR-363*	−5.43	−5.94	−4.90	−4.57	−1.28	−2.58
hsa-miR-326	−2.64	−3.15	−1.49	−1.04	−3.58	−3.85
hsa-miR-940	−4.53	−4.83	−3.95	−4.19	1.16	0.78
hsa-miR-125b-2*	−4.92	−4.76	−4.65	−4.50	−1.24	−0.04
hsa-miR-380-5p	−3.13	−4.64	−3.24	−3.31	−0.25	0.46
hsa-miR-892a	−3.04	−4.52	−5.31	−4.63	−1.21	−2.46
hsa-miR-297	−4.18	−4.51	−5.48	−4.80	0.39	−0.07
hsa-miR-567	−4.20	−4.43	−3.59	−3.83	−1.93	−0.85
hsa-miR-885-3p	−3.98	−4.17	−4.28	−4.53	−0.16	0.21
hsa-miR-555	−3.92	−3.97	−3.00	−2.98	−0.32	−0.28
hsa-miR-29c	−3.41	−3.92	−3.32	−3.41	−1.19	−2.79
hsa-miR-593	−4.21	−3.87	−4.39	−2.66	0.37	0.29
hsa-miR-665	−3.86	−3.85	−3.41	−4.20	0.91	−1.14
hsa-miR-34b*	−4.32	−3.82	−3.78	−4.07	1.64	1.59
hsa-miR-187-3p	−3.74	−3.81	−3.26	−3.13	−0.14	−0.91
hsa-miR-708	−3.05	−3.80	−2.77	−3.28	−2.94	−2.64
hsa-miR-506	−0.95	−3.14	−3.12	−2.17	−1.34	−2.47
hsa-miR-601	−2.90	−3.70	−2.98	−3.53	0.02	−1.08
hsa-miR-124a	−2.78	−2.50	−2.50	−2.51	−0.37	−1.46

Abbreviation: miRNA = microRNA.

luciferase activity of the construct containing the B7-H3 3′-UTR compared with scrambled miRNA-transfected controls (Figure 3), indicating that these miRNAs directly bind to the B7-H3 3′-UTR.

High miR-29c expression is associated with increased breast cancer-related survival. To determine the possible prognostic role of B7-H3-regulating miRNAs, we analysed whether their expression level correlates with breast cancer prognosis in two independent patient cohorts. Of the selected 20 miRNAs, 10 (miR-214, -940, -125b-2*, -885, -29c, -665, -34b*, -708, -601, and -326) were expressed in the tumour samples in both clinical cohorts and were included in the analyses. Distribution of the clinicopathological parameters of the patients in the two cohorts is given in Supplementary Table S2. Using breast cancer death as an end point, we first checked whether any of the 10 miRNAs were of significant prognostic importance. This was screened by separating the individual miRNA values in quartiles. The analysis was first performed in a cohort of 142 patients from DBCG82bc study (Kyndi *et al*, 2008; Myhre *et al*, 2010; Aure *et al*, 2013). One miRNA (miR-29c) was found to have prognostic value that was associated with a high miR-29c expression (Figure 4A). This was further explored by a spline analysis aimed to optimise the best cut point between high and low values. This was found to be a value of 3.35 (by coincidence almost identical with the cut point between the third and fourth quartile). On the basis of this cut point, we then performed a univariate analysis and found a hazard ratio (HR): 0.37 (0.20–0.69, 95% confidence level), with $P = 0.001$ for breast cancer death. After adjusting for age, tumour size, nodal status, HER2 and ER status, the HR was: 0.46 (0.25–0.86), $P = 0.008$. In addition, when we analysed miR-29c expression in relation to distant metastasis, we found that higher miR-29c expression was associated with lower risk of developing distant metastasis (HR: 0.48 (0.29–0.80)).

Next, we validated this finding in 81 stage I and II breast cancer patients (MicMa) (Enerly *et al*, 2011). Using the same approach as above, we independently confirmed the superior prognostic value of high miR-29c expression (Figure 4B) with a univariate HR of 0.39 (0.15–0.98), $P = 0.04$, and a multivariate adjusted HR of 0.36 (0.14–0.93), $P = 0.02$, for breast cancer death.

As we had demonstrated that miR-29c binds to B7-H3 3′-UTR and downregulates the B7-H3 protein in breast cancer cell lines, we investigated whether the observed association between high miR-29c and improved prognosis was related to the reduced level of B7-H3 in the tumours. When we analysed a potential correlation between B7-H3 transcript and miRNA expression levels using the Spearman correlation in the MicMa cohort, we found a significant negative correlation ($r = -0.268$; $P = 0.008$) between B7-H3 mRNA and miR-29c (Supplementary Figure S3). In the cell lines, endogenous miR-29c levels were low, whereas B7-H3 had relatively high expression (Supplementary Figure S4).

HER2 expression and loss of oestrogen receptor (ER) expression both indicate more aggressive breast cancer, whereas inactivation of the p53 tumour suppressor is common in several cancer forms including breast cancer. We therefore investigated whether any of these parameters were associated with miR-29c expression, by comparing the miR-29c mean value in *TP53* normal and mutated tumours, in HER2-positive and HER2-negative tumours and in ER-positive and ER-negative tumours. In both cohorts, we found that miR-29c had lower expression in tumours with *TP53* mutation (MicMa $P < 0.001$, DBCGbc $P = 0.048$), HER2 overexpression (MicMa $P = 0.037$, DBCGbc $P = 0.004$) and loss of ER expression ($P < 0.001$ both cohorts) (Supplementary Table S3). In addition, miR-29c had lower expression in higher grade tumours and in the basal subtype (Supplementary Figure S5), which fits well with the fact that higher miR-29c expression predicts better survival.

DISCUSSION

In this study, we present the results of a high-throughput miRNA gain-of-function screening, followed by protein LMA to identify and validate miRNAs that affect the expression of B7-H3 in breast cancer cells. Except for miR-29c, which has been reported to directly target B7-H3 in neuroblastomas, sarcomas and brain tumours (Xu *et al*, 2009) and in melanoma (Wang *et al*, 2013), and miR-187, which has been shown to target B7-H3 in renal cell carcinoma (Zhao *et al*, 2013), very little is known regarding B7-H3 and its regulation by miRNAs. This is the first study where the effect of an entire library of miRNAs on B7-H3 protein expression has been studied. We identified several miRNAs, including miR-29c, which directly targeted B7-H3 by binding to the 3′-UTR sequence, and demonstrated that miR-29c was significantly associated with increased survival in two cohorts of breast cancer patients.

In the initial screen, we identified nearly 50 miRNAs potentially downregulating B7-H3. The large number of hits reflects the well-known fact that there are multiple miRNAs targeting an individual gene. In addition, many miRNAs may target other mRNAs, which then indirectly may downregulate the B7-H3 protein expression.

In attempts to validate the 20 most efficient miRNAs downregulating B7-H3 protein in the screens, we found that all the selected miRNAs led to reduced B7-H3 protein expression in JIMT-1 cells. For the KPL-4 cells, the effects at the B7-H3 protein level were more variable and less pronounced for some of the

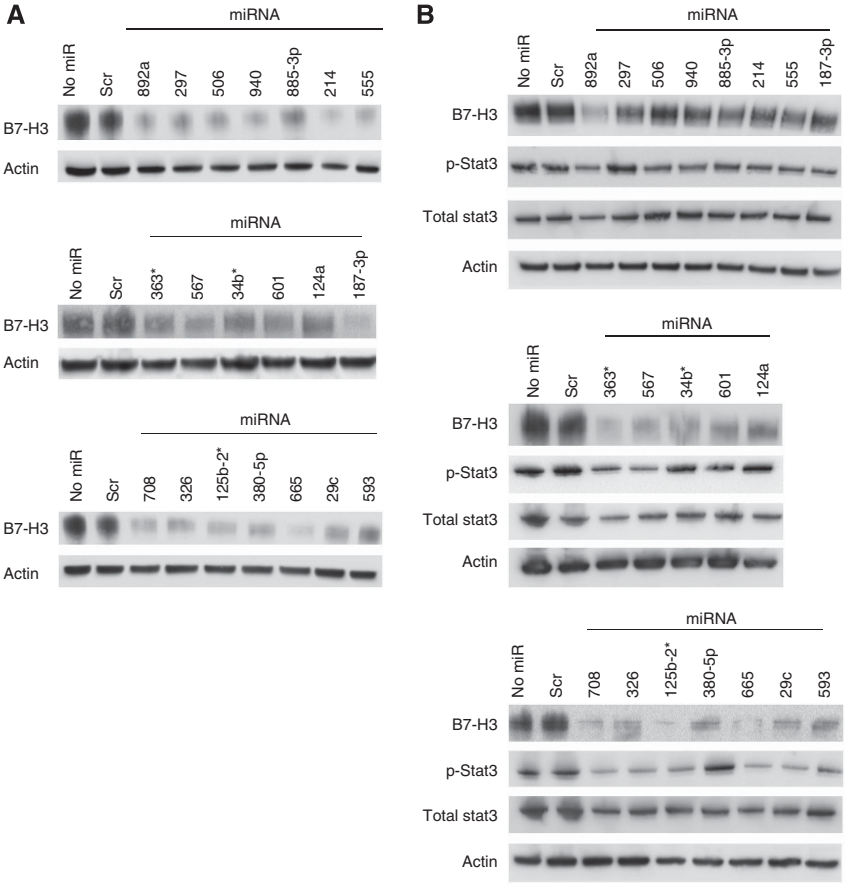

Figure 2. Validation of miRNAs regulating B7-H3. JIMT-1 (**A**) and KPL-4 (**B**) were transfected with miRVANA constructs (20 nM) and incubated for 72 h. Thereafter, the cells were lysed and the lysates were analysed by western immunoblotting for the expression of B7-H3 (**A** and **B**) and p-Stat3 (**B**). The expression of β-actin was used as a loading control. Scr, scrambled miRNA. The experiments were repeated three (JIMT-1) or two (KPL-4) times, and representative western blots are shown.

Table 2. Predicted binding sites in the B7-H3 3′-UTR for the miRNAs selected for validation

miRNA	Position in the B7-H3 3′-UTR	Prediction algorithm
hsa-miR-214	188–194 and 257–263	miRanda/TargetScan
hsa-miR-363*	374–380	TargetScan
hsa-miR-326	1117–1123	miRanda/TargetScan
hsa-miR-940	253–259, 398–404 and 1035–1041	miRanda/TargetScan
hsa-miR-892a	272–278 and 295–302	TargetScan
hsa-miR-297	278–284	miRanda/TargetScan
hsa-miR-567	278–284	TargetScan
hsa-miR-555	1113–1120	miRanda/TargetScan
hsa-miR-29c	1339–1346	miRanda/TargetScan
hsa-miR-665	544–551, 1079–1085 and 1390–1396	TargetScan
hsa-miR-34b*	468–474	TargetScan
hsa-miR-187-3p	416–423	miRanda/TargetScan
hsa-miR-708	35–42 and 1388–1394	miRanda/TargetScan

Abbreviation: miRNA = microRNA.

miRNAs, for example, miR-506 and miR-187-3p. MiR-506 was not predicted to bind directly to B7-H3 3′-UTR, suggesting that it targets B7-H3 through an indirect mechanism in the JIMT-1 cell line, which may not be functioning in KPL-4 cells. Notably, several of the miRNAs that reduced B7-H3 protein levels in KPL-4 cells caused reduced phosphorylation of Stat3. Previously, it was shown that B7-H3 knockdown leads to decreased activation of Stat3 (Liu *et al*, 2011). MiR-892a and miR-363* clearly reduced the levels of both total Stat3 and p-Stat3, suggesting that these miRNAs might target Stat3 directly, although Stat3 was not predicted to be a direct target for these two miRNAs by two widely used target prediction algorithms, miRanda and TargetScan.

Using luciferase assays, we showed that 13 miRNAs: miR-214, miR-363*, miR-326, miR-940, miR-29c, miR-665, miR-34b*, miR-708, miR-601, miR-124a, miR-380-5p, miR-885-3p, and miR-593 directly target B7-H3 by associating with the B7-H3 3′-UTR region, suggesting that these miRNAs have a direct role in modulating the B7-H3 protein expression. The fact that miR-124a, miR-380-5p, miR-885-3p and miR-593 were not predicted to bind B7-H3 3′-UTR by neither TargetScan nor miRanda, demonstrates the limitation of prediction programs and the importance of experimental validation. Interestingly, we also found that several miRNAs reduced the protein expression without binding to B7-H3 3′-UTR. This may indicate that B7-H3 can be indirectly regulated by other target genes, or that these miRNAs do not target B7-H3 in the 3′-UTR but rather in other regions. It has been reported that several miRNAs exert their control on mRNAs through target sites that reside beyond the 3′-UTR region (Lytle *et al*, 2007; Orom *et al*, 2008; Tay *et al*, 2008; Leivonen *et al*, 2011).

In both the DBCGbc cohort and the validating MicMa cohort, breast cancer-related survival was significantly increased in patients with high expression of miR-29c. In the DBCGbc cohort, higher miR-29c expression was also associated with lower risk of

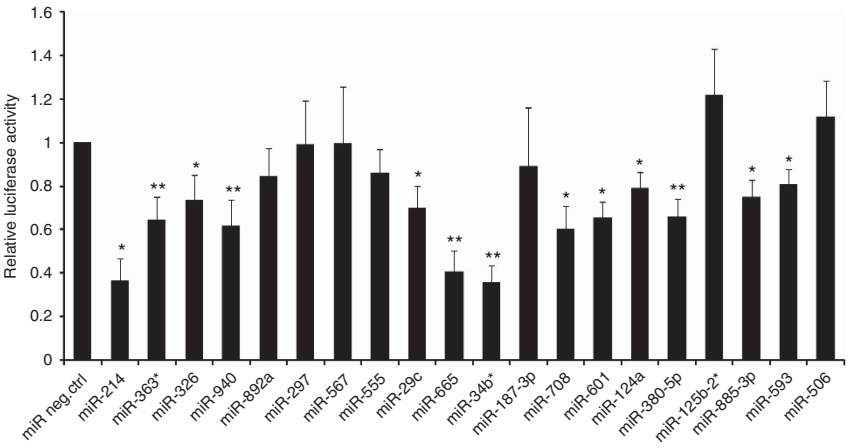

Figure 3. B7-H3 is a direct target for miR-214, miR-363*, mir-326, miR-940, miR-29c, miR-665, miR-34b*, miR-708, miR-601, miR-124a, miR-380-5p, miR-885-3p and miR-593. Activity of the luciferase reporter containing the B7-H3 3′-UTR. Reporter (100 ng) was co-transfected with miRVANA constructs (50 nM) for the indicated miRNAs, and the luciferase activity was measured after 24 h incubation. The experiments were repeated at least three times with triplicates. *$P<0.05$, **$P<0.01$.

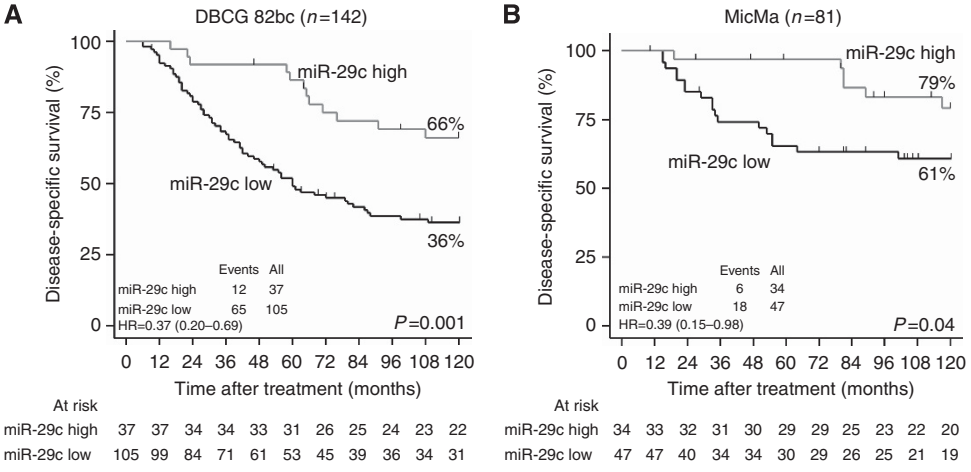

Figure 4. Kaplan–Meier survival curves presenting disease-specific survival (A, DBCGbc cohort; B, MicMa cohort) based on high or low expression of miR-29c.

developing distant metastasis. Several reports have demonstrated miR-29c to be important in the progression and prognosis for various cancers (Xiong *et al*, 2010; Zhao *et al*, 2010; Nguyen *et al*, 2011; Presneau *et al*, 2013), and that miR-29c enhances the sensitivity of nasopharyngeal carcinoma to cisplatin-based chemotherapy and radiotherapy (Zhang *et al*, 2013). To our knowledge, the present study is the first demonstrating a clinical impact of miR-29c in patients with breast cancer. It is tempting to speculate that this may at least partly be owing to reduced B7-H3 protein expression in these patients' tumours via a direct regulation of B7-H3 by miR-29c, and we have previously shown that B7-H3 is involved in sensitivity to chemotherapy and metastasis formation (Liu *et al*, 2011; Tekle *et al*, 2012). Interestingly, in the MicMa cohort, we found significant inverse correlation between the levels of B7-H3 transcript and miR-29c, indicating that miR-29c might affect B7-H3 expression also *in vivo*.

Although the other miRNAs that we investigated had no significant impact on breast cancer-related survival, circulating miR-125b-2* was reported to be a marker predicting chemoresistance in patients with invasive ductal carcinoma (Wang *et al*, 2012), and miR-34b* expression correlated negatively with disease-free survival and overall survival in triple-negative breast cancer patients (Svoboda *et al*, 2012). Furthermore, several of the other miRNAs have been linked to cancer progression and prognosis in other tumour types (Kefas *et al*, 2009; Saini *et al*, 2011; Jang *et al*, 2012; Majid *et al*, 2012; Shaham *et al*, 2012; Svoboda *et al*, 2012; Xia *et al*, 2012).

We also found that low expression of miR-29c was associated with *TP53* mutation in both cohorts of breast cancer patients. MiR-29 has previously been linked to stabilisation of the p53 protein by targeting p85α (Park *et al*, 2009). Accordingly, tumours with low miR-29c expression frequently have lost the TP53 tumour suppressor activity, and low miR-29-expressing tumours with wild-type *TP53* gene might, nevertheless, have reduced TP53 activity owing to destabilisation of the protein. We also found that low miR-29c was associated with overexpression of HER2 and loss of ER expression in both cohorts. Neither HER2 expression nor loss of ER expression has previously been linked to miR-29c. However, as both are features of aggressive breast cancer, their association with low miR-29c corresponds well with our disease-specific survival data (high miR-29c expression predicts prolonged survival). In addition, the expression of miR-29c was significantly lower in higher grade tumours compared with lower grade tumours. miR-29c also had the lowest expression in the basal subtype, which is the most aggressive form of breast cancer.

The molecular basis for the role of B7-H3 in tumour biology needs to be further elucidated, the importance of which is illustrated by the many reports showing that its expression is linked to a worsened prognosis in several cancer forms. Most studies have focused on B7-H3 and its role as an immunoregulatory molecule. We, however, have reported that B7-H3 has important roles in chemoresistance and metastasis independently of the immune system. On the basis of emerging evidence for miRNAs as regulators of cancer metastasis, it is interesting that we have identified several miRNAs targeting B7-H3 and also demonstrated significant clinical impact of one of these, miR-29c.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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