VGLL1 expression is associated with a triple-negative basal-like phenotype in breast cancer

María Ángeles Castilla^{1,2}, María Ángeles López-García^{1,2}, María Reina Atienza¹, Juan Manuel Rosa-Rosa³, Juan Díaz-Martín^{1,2}, María Luisa Pecero¹, Begoña Vieites^{1,2}, Laura Romero-Pérez^{1,2}, Javier Benítez⁴, Annarica Calcabrini⁵ and José Palacios^{2,3}

¹Instituto de Biomedicina de Sevilla–CSIC–Universidad de Sevilla, Hospital Universitario Virgen del Rocío, Department of Pathology, Avda. Manuel Siurot S/N, 41013 Seville, Spain

²Red Temática de Investigación Cooperativa en Cáncer (RTICC), ISCIII, Madrid, Spain

³Servicio de Anatomía Patológica, Hospital Universitario Ramón y Cajal and Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain

⁴Human Genetics Group, Departamento de Biología del Cáncer, Spanish National Cancer Research Centre (CNIO)–CIBERER, Madrid, Spain

⁵Department of Technology and Health, Istituto Superiore di Sanità, Rome, Italy

Correspondence should be addressed to J Palacios or M Á Castilla
Emails
jose.palacios@salud.madrid.org or macastillamoro@gmail.com

Abstract

Vestigial-like 1 (*VGLL1*) is a poorly characterized gene encoding a transcriptional co-activator structurally homologous to *TAZ* and *YAP* that modulates the Hippo pathway in *Drosophila*. In this study, we examined the expression of *VGLL1* and its intronic miRNA, miR-934, in breast cancer. *VGLL1* and miR-934 expression miRNA profiling was carried out on frozen samples of grade 3 invasive ductal carcinomas. *VGLL1* protein was also examined in 433 sporadic and *BRCA1*-associated breast carcinomas on tissue microarrays. RNA-seq data from The Cancer Genome Atlas (TCGA) was used to confirm differences in *VGLL1* and miR-934 expression in different breast cancer subtypes, and to correlate their expression with that of other genes and miRNAs. Of 28 miRNAs differentially expressed in estrogen receptor (ER)-positive and ER-negative grade 3 breast carcinomas, miR-934 was most strongly upregulated in ER-negative carcinomas, and its expression was correlated with that of *VGLL1*. Nuclear *VGLL1* expression was observed in 13% of sporadic breast carcinomas, and while *VGLL1* was only occasionally found in luminal A (0.70%) and B (5.60%) carcinomas, it was often expressed in HER2-positive (17%), triple-negative (TN) breast carcinomas (>40%) and *BRCA1*-associated TN carcinomas (>50%). These findings were confirmed in the TCGA dataset, which revealed positive associations with luminal progenitor genes (*GABRP*, *SLC6A14*, *FOXC1*, *PROM1*, and *BBOX1*) and strong negative correlations with ER-associated genes (*ESR1*, *C6ORF211*, *GATA3*, and *FOXA1*). Moreover, *VGLL1* expression was associated with reduced overall survival. In conclusion, *VGLL1* and miR-934 are mainly expressed in sporadic and *BRCA1*-associated TN basal-like breast carcinomas, and their coordinated expression, at least partially mediated by the direct modulation of *ESR1*, might be involved in the maintenance of a luminal progenitor phenotype.

Key Words

- breast cancer
- Vgll1
- miR-934
- basal-like phenotype
- BRCA1

Endocrine-Related Cancer
(2014) 21, 587–599

Introduction

Breast cancer is a heterogeneous disease characterized by marked variation in morphological appearance, molecular features, biological behavior, and therapeutic response. Based on the results of gene expression studies, breast carcinomas can be divided into different molecular subtypes with clinical relevance: luminal (A and B), HER2+, and basal-like breast cancer (Perou et al. 2000, Sorlie et al. 2001, 2003). Although there is significant morphological and molecular heterogeneity among human basal-like breast cancers, most are high-grade, aneuploid, invasive carcinomas, which do not express estrogen receptor (ER) and/or progesterone receptor (PR), or HER2, and that frequently exhibit TP53 mutations (80%). Moreover, the loss of RB1 and BRCA1 are characteristic features in basal-like tumors (2012). Although complete concordance is lacking, basal-like breast carcinomas are identified pathologically as triple-negative (TN) tumors (i.e. immunohistochemically negative for ER, PR, and HER2) that express some basal markers, such as basal cytokeratins (CK5/6, CK14, and CK17) and/or EGFR (Badve et al. 2011, Toft & Cryns 2011). Basal-like carcinomas have a worse prognosis compared with other breast tumor subtypes and cannot be treated using ER- or HER2-targeted therapies (reviewed in Toft & Cryns (2011)).

During the course of an ongoing study into the pattern of miRNA expression in grade 3 invasive breast cancer, we observed marked overexpression of miR-934 in TN breast tumors. The function of this miRNA is unknown, but it is located in intron 4 of the vestigial-like 1 (VGLL1) gene. VGLL1 is a human protein that is related to the *Drosophila* transcriptional co-activator Vg. VGLL1, which is located on Xq26.3, was identified and isolated from a fetal heart cDNA library and named *Tondu* (TDU; Vaudin et al. 1999). The homology between Vg and TDU is limited to a short domain of 26 amino acids that is essential for its interaction with TEADs (a family of transcriptional factors related to sd – scalloped – in *Drosophila*). Because Vg and TDU have few similarities, TDU is not considered to be orthologous to Vg, even though functional studies have shown that TDU can rescue Vg function, suggesting that these proteins can fulfill analogous functions (Vaudin et al. 1999). In addition, given that TDU can interact with all TEADs, it seems that TDU is indeed a TEAD co-activator (Vaudin et al. 1999).

Although the role of VGLL1 in both normal development and disease is unknown in mammals, some

indication of the function of VGLL1 may be found by analyzing Vg, a transcription factor required for wing cell specification in flies. Non-wing cells are recruited after repression of the Hippo pathway, resulting in the nuclear accumulation of Yki (TAZ and YAP in mammals). Yki forms a complex with Sd (TEADs factors in mammals) that directly activates Vg, triggering the conversion of non-wing cells into wing cells. Interestingly, as Vg accumulates, it can substitute Yki as a Sd co-factor that drives Vg gene expression (Zecca & Struhl 2010). It is not known whether similar relationships govern the development of mammalian tissues.

Structural and functional similarities were recently described between the VGLL1–TEAD complex and the complex formed by TEAD and YAP, demonstrating that VGLL1 acts as a specific TEAD co-activator in a manner similar to that of the oncogene YAP (Pobbati et al. 2012). Through his TEAD-interaction domain, YAP enhances multiple processes known to be important for tumor progression and metastasis, including cell proliferation, transformation, migration, and invasion (Lamar et al. 2012). Similarly, it was demonstrated that VGLL1–TEAD complexes promote anchorage-independent cell proliferation and the upregulation of specific proliferation genes *in vitro*, suggesting a role of VGLL1 in cancer initiation and/or progression (Pobbati et al. 2012). However, no further *in vitro* or *in vivo* studies have specifically been analyzed for the role of VGLL1 in human cancer.

Based on the observed overexpression of miR-934 in TN breast cancer and the location of this miRNA in intron 4 of VGLL1 gene, we analyzed VGLL1 expression in a relatively large cohort of breast cancer patients. In this study, we describe the correlation between VGLL1 and miR-934 expression in breast cancer, and we demonstrate that VGLL1 nuclear protein is more frequently expressed in both sporadic and hereditary forms of TN basal-like breast carcinomas. Studies of several expression databases confirmed our observations and suggest an oncogenic role of VGLL1 in breast cancer.

Materials and methods

Human tumors

For miRNA and mRNA analyses, we obtained fresh-frozen samples from 60 grade 3 invasive ductal carcinomas (IDCs): 37 ER+ and 23 TN breast carcinomas (additional file 2, [Supplementary Table 1](#), see section on

supplementary data given at the end of this article). For immunohistochemical analysis, we used 349 formalin-fixed paraffin-embedded (FFPE) invasive human breast carcinomas (Table 1). These tumors had been diagnosed between 2006 and 2011, and inclusion criteria were availability of pathological data and tissue for tissue microarray (TMA) construction. To confirm the immunohistochemistry (IHC) results in this series, we obtained FFPE samples from 70 additional TN breast carcinomas (additional file 2, Supplementary Table 2) and 14 *BRCA1*-associated hereditary breast carcinomas (additional file 2, Supplementary Table 3). All samples, except the *BRCA1*-associated carcinomas (Palacios *et al.* 2005), were obtained from the archives of the Department of Pathology, Hospital Virgen del Rocío, Sevilla (Spain). In addition to tumor samples, we used five non-neoplastic breast tissue samples to analyze the immunohistochemical expression pattern of *VGLL1* in normal breast.

This study was performed in accordance with standard ethical procedures dictated by Spanish law (Ley de Investigación Orgánica Biomédica, 14th July 2007) and was approved by the Ethics Committee of the Hospital Virgen del Rocío and the Fundación Pública Andaluza para la Gestión de la Investigación en Salud de Sevilla (FISEVI), Spain. Written informed consent was obtained from all patients and all clinical analyses were conducted in accordance with the principles of the Helsinki Declaration.

Breast cancer cell lines

Human breast carcinoma cell lines with different phenotypes (MCF7, MCF10, T47D, BT474, SKBR3, MDA-MB-231, and BT459) were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) and were cultured according to the indicated supplier conditions. The cells were maintained as monolayer cultures at 37 °C in an atmosphere with 5% CO₂. Total RNA from breast carcinoma cell lines was extracted in triplicate from 90% confluent cells.

Expression of miRNAs and *VGLL1* mRNA

Total RNA was extracted from human breast carcinoma cell lines and selected areas of frozen tissue containing > 70% tumor cells using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) after having homogenized the tissue for 2 min in lysis buffer using an Ultra-turrax (T10 Basic, IKA, Staufen, Germany), following the manufacturer's protocol. The quantity and quality of the RNA

isolated were measured using a NanoDrop ND-100 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

The expression of 667 miRNAs was measured by quantitative real-time PCR (qRT-PCR) using customized TaqMan Human miRNA assays (Human MicroRNA card set v2.0, Applied Biosystems). To validate miR-934 expression, we used qRT-PCR with a TaqMan probe specific for the mature form of this miRNA (Applied Biosystems TaqMan MicroRNA Assays). Details of qRT-PCR have been previously described (Castilla *et al.* 2011, 2012) and are provided as Supplementary information, see section on supplementary data given at the end of this article (additional file 1).

The significance of relative changes in miRNA and mRNA expression between different breast tumor types was determined using the Wilcoxon's rank-sum test, and the *P* values were corrected using the Benjamini–Hochberg algorithm (*P* < 0.05). These analyses were performed with the Integromics RealTime StatMiner 4.2 Package (Integromics S.L., Granada, Spain), while SPSS (SPSS 17.0, Inc.) was used to perform the Pearson correlation analyses to determine the correlation between *VGLL1* and miR-934 gene expression.

Expression of miR-934 and *VGLL1* mRNA in The Cancer Genome Atlas dataset

To validate our results, we used The Cancer Genome Atlas (TCGA) RNA-seq data from the subset of invasive breast carcinomas, which consists of over 500 breast tumor samples. We downloaded the files containing the per-million read count from the TCGA data portal (<https://tcga-data.nci.nih.gov/tcga/findArchives.htm>) and grouped tumors into subtypes according to the Pam50 signature (basal-like, HER2-enriched, luminal A, luminal B, and normal-like; Supplementary Table 4, see section on supplementary data given at the end of this article).

As no normal tissue match was sequenced for any of the tumors, we normalized the data to allow miRNA and gene samples to be compared (referred to hereafter as locus/loci). Normalization was based on the background expression of the corresponding sample. By using the median of the expressed loci (count > 0) as the expression threshold for each sample, the fold-change in expression in the context of the tumor was obtained by dividing the value for each locus count by the sample threshold. We calculated the median expression for each locus in every subtype and for the global sample set. As the distribution of fold-change expression values was generally skewed (data not shown), the Kruskal–Wallis test was applied

Table 1 Association between VGLL1 protein expression and clinicopathological characteristics of infiltrating breast carcinomas

	Analyzable	VGLL1-positive (n=46)	VGLL1-negative (n=303)	P value*
Age (years)	58.99 ± 13.54	55.61 ± 14	59.51 ± 13.45	
Histologic type (n=349)				
Ductal carcinoma	310	42 (13.54%)	268 (86.45%)	0.7996
Lobular carcinoma	16	2 (12.50%)	14 (87.50%)	
Other	23	2 (8.69%)	21 (91.30%)	
pT (tumor size) (n=344)				
pT1	149 (44.31%)	19 (12.80%)	130 (87.20%)	0.9760
pT2	162 (47.09%)	22 (13.60%)	140 (86.40%)	
pT3	22 (6.40%)	3 (13.60%)	19 (86.40%)	
pT4	11 (3.20%)	1 (9.10%)	10 (90.90%)	
Nodal status (n=347)				
N negative	181 (52.16%)	20 (11.00%)	161 (89.00%)	0.2666
N positive	166 (47.83%)	25 (15.10%)	141 (84.90%)	
Pathological stage (n=344)				
I	108 (31.40%)	12 (10.40%)	96 (88.90%)	0.8180
II	146 (42.44%)	18 (15.10%)	128 (87.70%)	
III	82 (23.84%)	13 (15.90%)	69 (84.10%)	
IV	8 (2.33%)	1 (12.50%)	7 (87.50%)	
Histological grade (n=348)				
Grade 1	44 (12.61%)	0 (0.00%)	44 (100.00%)	<0.0001
Grade 2	119 (34.12%)	7 (5.90%)	121 (94.10%)	
Grade 3	185 (53.30%)	39 (21.11%)	146 (78.90%)	
KI-67 (n=349)				
KI-67 <14%	170 (48.71%)	3 (1.80%)	167 (98.20%)	<0.0001
KI-67 14–50%	137 (39.26%)	24 (17.50%)	113 (82.50%)	
KI-67 ≥50%	42 (12.03%)	19 (45.20%)	23 (54.80%)	
Immunohistochemical markers				
ER (n=349)				
Negative	118 (33.80%)	36 (30.50%)	82 (69.50%)	<0.0001
Positive	231 (66.18%)	10 (4.30%)	213 (95.70%)	
PR (n=347)				
Negative	154 (44.30%)	39 (25.30%)	115 (74.70%)	<0.0001
Positive	193 (56.70%)	7 (3.60%)	186 (96.40%)	
HER2 (n=348)				
Negative	259 (74.42%)	31 (12.00%)	228 (88.80%)	0.2405
Positive	89 (25.57%)	15 (16.90%)	74 (83.10%)	
EGFR (n=347)				
Negative	328 (94.62%)	40 (12.20%)	288 (87.80%)	0.0749
Positive	19 (5.48%)	5 (26.30%)	14 (73.70%)	
Basal CKs (n=348)				
Negative	311 (89.36%)	28 (9.00%)	283 (91.00%)	<0.0001
Positive	37 (10.60%)	18 (48.60%)	19 (51.40%)	
c-KIT (n=312)				
Negative	298 (95.55%)	33 (11.07%)	264 (88.93%)	<0.0002
Positive	14 (4.45%)	7 (50%)	7 (50%)	
Molecular phenotype (n=349)				
Luminal A	142 (40.70%)	1 (0.70%)	141 (99.30%)	<0.0001
Luminal B	54 (15.50%)	3 (5.60%)	51 (94.40%)	
HER2+/RE+	28 (8.80%)	5 (17.90%)	23 (82.10%)	
HER2	58 (16.60%)	10 (17.20%)	48 (82.80%)	
TN-NOS	32 (9.20%)	11 (34.40%)	21 (65.60%)	
TN-BL	35 (10.00%)	16 (45.70%)	19 (54.30%)	

ER, estrogen receptor; PR, progesterone receptor; EGFR, epidermal growth factor receptor; basal CKs, basal cytokeratins (CK5/6 and/or CK17); TN-NOS, triple-negative not otherwise specified; TN-BL, triple-negative basal-like. *P values were calculated using the Pearson's χ^2 test. Bold, P values are statistically significant.

to determine differences between subtypes, followed by Mann–Whitney *U* and Wilcoxon's tests for subtype pairwise analyses. A Pearson's correlation was used to confirm the correlation between *VGLL1* and miR-934 expression, as a linear relationship was expected between the two loci. However, a Spearman's correlation was used when interactions between other miRNAs and genes were analyzed, as a monotonic relationship was expected in these cases. Statistics were performed using in-house perl (<http://www.perl.org/>) scripts and R (<http://www.r-project.org/>).

To determine whether a given miRNA could directly interact with a gene, we used data from the microRNA.org database (<http://www.microrna.org/microrna/getDownloads.do>) which contains published data and several available predictors (Dweep et al. 2011). We evaluated three available predictors (miRNA.org values) considered to be the best tools for making predictions: conservation, energy, and mirSVR score. To determine whether our observations at the mRNA level had a measurable effect at the protein level, we obtained contrast results when compared with those data from the cBioPortal protein array (Cerami et al. 2012), bearing in mind that these arrays contain a limited number of proteins.

In addition, CNA status was checked (Cerami et al. 2012) on 71 samples overexpressing *VGLL1* to confirm or discard the association between increased number of copies of 200 kb region surrounding *VGLL1* in chromosome X and overexpression of *VGLL1* and miR-934.

TMA construction and IHC

Tissue sections from FFPE samples were stained with hematoxylin and eosin and representative areas were selected by three pathologists (J Palacios, M Á Castilla, and B Vieites) to obtain two 1 mm diameter tissue cores. Different TMAs were constructed with invasive breast carcinomas. IHC was carried out on TMA sections using the Envision method (Dako, Glostrup, Denmark) using primary antibodies against ER, PR, HER2, Ki-67, CK5/6, CK17, EGFR, CKIT, p63, CD3, CD20, and CD45. Myoepithelial staining of p63 was carried out to verify the diagnosis of ductal carcinoma *in situ*. CD3, CD20, and CD45 were used to identify intraepithelial and stromal lymphocytes. Details of the clones, suppliers, dilutions and scoring criteria used are provided in additional file 2 (Supplementary Table 5, see section on supplementary data given at the end of this article). Details for microscope, camera, software, and conditions used to capture IHC images are provided as Supplementary information (additional file 1).

Based on recent recommendations (Goldhirsch et al. 2011), breast carcinomas were classified into five IHC-based subtypes as follows: luminal A (ER+ and/or PR+, HER2–, Ki-67 index <14%); luminal B/HER2– (ER+ and/or PR+, HER2–, Ki-67 index ≥14%); luminal B/HER2+ (ER+ and/or PR+, HER2+); HER2 (ER–, PR–, and HER2+); and TN (ER–, PR–, and HER2–). TN breast carcinomas that expressed any basal cytokeratin and/or EGFR were termed TN-BL (basal-like), while all other TN breast carcinomas were termed TN-NOS (not otherwise specified).

VGLL1 protein expression was assessed by IHC using the Envision method (Dako) and VGLL1 rabbit polyclonal antibody (10124-2-AP, Proteintech, Chicago, IL, USA) at 1:50 dilution, after heat-induced retrieval step. Following provider's recommendation, we used prostate carcinomas as positive control.

Vgl1 IHC data and clinicopathological parameters were analyzed using the Pearson's χ^2 cross-tabulation test. The *P* value for the total effect of a factor was calculated from the (partial) likelihood ratio statistic. Analyses were performed with the JMP 8.0 Software Package (JMP Statistical Discovery Software, SAS Institute, Inc., Cary, NC, USA).

Immunofluorescence

SKBR3 and MCF7 breast cancer cells were seeded on sterile coverslips, fixed with 4% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100/PBS, and blocked with 10% normal goat serum/PBS. Coverslips were incubated for 2 h at 37 °C with a rabbit polyclonal anti-VGLL1 (Proteintech; 1:50 in PBS–1% BSA), then with a goat anti-rabbit IgG Alexa-Fluor 488 (1:400 in PBS–1% BSA) for 1 h at 37 °C. After washing with PBS, the cells were counterstained with 10 µg/ml DAPI/PBS (10 min at 37 °C) and coverslips were mounted on slides with ProLong reagent (Life Technologies). The samples were observed and photographed using a Nikon Eclipse 90i fluorescence microscope equipped with Digital Sight DS-Qi1 camera and Nis-Elements BR imaging software (Nikon, Tokyo, Japan).

Survival analysis

Survival analysis was performed on a group of 212 invasive breast carcinoma patients for whom clinical data were available. The details concerning to therapy received are provided as Supplementary information (additional file 1).

The probabilities of overall survival (OS) and disease-free survival (DFS) were estimated using the Kaplan–Meier method and survival differences determined by the log-rank test. Univariate and multivariate analyses of OS and DFS were performed using the Cox proportional hazard model (SPSS 17.0, SPSS, Inc.). OS was defined as the period between the first diagnostic biopsy and death caused by breast cancer-related complications, and DFS as the period between the first diagnostic biopsy and the first local or distant recurrence/relapse.

Results

Expression of miR-934 and VGLL1 mRNA in grade 3 IDCs

We first investigated miRNAs that were differentially expressed between 22 ER+ and 13 TN grade 3 fresh frozen IDCs. We examined a total of 667 miRNAs in these samples by quantitative RT-PCR using TaqMan miRNA arrays. Expression analysis revealed 28 miRNAs that were differentially expressed between the two groups (false discovery rate (FDR) <0.05), 14 miRNAs that were upregulated and 14 downregulated in TN breast carcinomas. In all cases, at least a twofold difference in expression was detected between the two groups (Table 2).

Interestingly, we identified six upregulated miRNAs that were localized on chromosome X in TN IDCs (Table 2), four of them being located in the Xq26–28 band (miR-934, miR-505, miR-505*, and miR-18b). The miR-934 was the most strongly upregulated miRNA in TN IDCs (61.5-fold increase with respect to ER+ breast carcinomas). The large majority of TN IDCs analyzed expressed miR-934; however, this miRNA was detected in only two of the 22 grade 3 ER+ IDCs, in which it was expressed at very low levels.

To validate the upregulation of miR-934 observed in TN IDCs, we analyzed a larger number of fresh-frozen tumor samples. In total, we analyzed 60 grade 3 IDCs by qRT-PCR using a TaqMan probe specific for miR-934, 37 ER+, and 23 TN IDCs. Consistent with our initial result, we observed a significant increase in the expression of miR-934 in TN as opposed to ER+ IDCs ($P=0.013$; Fig. 1a).

As miR-934 is embedded in intron 4 of the *VGLL1* gene, we investigated whether *VGLL1* expression also varied between these subtypes of grade 3 IDCs. Analysis of *VGLL1* mRNA expression by qRT-PCR revealed a marked increase in gene expression in TN (15.66-fold increase, $P=0.001$) vs ER+ IDCs (Fig. 1b). Moreover, mRNA *VGLL1* expression was directly correlated with miR-934 expression (Pearson's $r=0.611$, $P<0.001$; Fig. 1c).

Table 2 Dysregulated miRNA expression in triple-negative vs luminal grade 3 breast carcinoma

miRNA	Fold-change	Adj. <i>P</i> value	Chromosome localization
miR-934	61.50	3.50×10^{-3}	Xq26.3
miR-505	25.89	4.00×10^{-2}	Xq27.1
miR-135b-3p	15.70	2.56×10^{-2}	1q32.1
miR-135b-5p	9.38	4.35×10^{-2}	1q32.1
miR-9-3p	7.54	1.44×10^{-2}	1q22
miR-9-5p	7.29	4.85×10^{-2}	1q22
miR-25*	7.11	4.84×10^{-2}	7q22.1
miR-545*	5.35	2.94×10^{-2}	Xq13.1
miR-505*	5.21	2.93×10^{-2}	Xq27.1
miR-18b	3.12	4.00×10^{-2}	Xq26.2
miR-628-3p	3.06	2.93×10^{-2}	15q21.3
miR-18a	2.92	4.00×10^{-2}	13q31.3
miR-146a	2.41	4.00×10^{-2}	5q34
miR-660	2.09	4.00×10^{-2}	Xq11.23
miR-190b	−13.25	3.50×10^{-3}	15q22.2
miR-337-5p	−12.14	9.74×10^{-3}	14q32.3
miR-943	−9.42	2.93×10^{-2}	4q16.3
miR-526b*	−7.35	3.92×10^{-2}	19q13.42
miR-149	−5.88	1.65×10^{-2}	2q27.3
miR-29c*	−4.52	2.93×10^{-2}	1q32.2
miR-342-3p	−3.15	4.35×10^{-2}	14q32.2
let-7b	−2.73	4.35×10^{-2}	22q13.31
miR-29c	−2.44	4.64×10^{-2}	1q32.2
miR-425	−2.40	4.35×10^{-2}	3p21.31
miR-103	−2.30	4.35×10^{-2}	5q34
miR-126*	−2.06	1.73×10^{-2}	9q34.3
miR-135a*	−2.05	2.97×10^{-2}	3q21.1
let-7f	−2.01	4.47×10^{-2}	Xq11.22

miRNA*, miRNA expressed at low levels relative to the same miRNA in the opposite arm of a hairpin. The significance of the relative changes in miRNA expression between grade 3 breast tumors was determined using the Wilcoxon's rank-sum test. *P* values were corrected using the Benjamini–Hochberg algorithm ($P<0.05$).

Expression of miR-934 and VGLL1 in the TCGA dataset

The change in the expression observed in the RNA-seq data confirmed the differential expression of miR-934 and *VGLL1* among breast cancer subtypes (additional file 2, [Supplementary Table 6](#), see section on [supplementary data](#) given at the end of this article). Expression of *VGLL1* and miR-934 was over 100 times higher in basal-like carcinomas than in all other subtypes. It should be noted that the expression values for both loci were close to 0 for HER2-enriched and luminal A and luminal B tumors, while they were close to twice the tumor background threshold in basal-like tumors. Pairwise analyses (additional file 2, [Supplementary Table 7](#)) revealed that the basal-like subtype differed significantly from HER2-enriched, luminal A, and luminal B subtypes. Pairwise results appeared to be biased for the normal-like subtype, probably due to the small number of tumors analyzed, and

Figure 1

miR-934 and *VGLL1* mRNA expression in breast cancer. Expression of miR-934 (upper panel, A) and *VGLL1* mRNA (middle panel, B) determined by quantitative RT-PCR in fresh-frozen samples of grade 3 estrogen receptor-positive and triple-negative breast carcinomas. The significance of relative changes was determined by the Wilcoxon's rank-sum test and the *P* values were corrected using the Benjamini-Hochberg algorithm ($P < 0.05$). The Y-axis shows fold changes in relative gene expression. (C) Correlation between miR-934 and *VGLL1* expression levels. Pearson correlation coefficients (*R*) and the level of statistical significance (*P*) of the correlations were determined.

for the HER2-enriched, luminal A, and luminal B subtypes, probably due to their low levels of expression. There was a significant correlation between *VGLL1* and miR-934 expression in this series (Pearson's $r = 0.86$, $P < 2.2 \times$

10^{-16}), confirming our previous observations. It is important to highlight that none of the 71 samples overexpressing *VGLL1* and miR-934 showed alterations in the number of copies of the region surrounding *VGLL1*.

To determine which genes are candidate targets to be regulated by miR-934 in breast cancer, we assessed the Spearman's correlation between miR-934 and all the genes enclosed in the dataset (not shown). *VGLL1* was most strongly correlated with miR-934 (Table 3), supporting the hypothesis of simultaneous transcription. Interestingly, ER (*ESR1*), *FOXA1*, and *GATA3*, which are thought to

Table 3 Top 10 genes most positively and negatively correlated with *VGLL1* and miR-934 expression

Gene	Spearman's correlation	P value
Genes correlated to miR-934 expression		
<i>VGLL1</i>	0.7824696	2.2×10^{-16}
<i>GABRP</i>	0.6591587	2.2×10^{-16}
<i>SFRP1</i>	0.6065420	2.2×10^{-16}
<i>PROM1</i>	0.5941352	2.2×10^{-16}
<i>ROM1</i>	0.5941352	2.2×10^{-16}
<i>BCL11A</i>	0.5871717	2.2×10^{-16}
<i>FOXC1</i>	0.5845652	2.2×10^{-16}
<i>FABP7</i>	0.5829631	2.2×10^{-16}
<i>PPP1R14C</i>	0.5759719	2.2×10^{-16}
<i>BBOX1</i>	0.5753999	2.2×10^{-16}
<i>FOXA1</i>	-0.5591262	2.2×10^{-16}
<i>C6ORF211</i>	-0.5363153	2.2×10^{-16}
<i>ESR1</i>	-0.5284415	2.2×10^{-16}
<i>GATA3</i>	-0.5224655	2.2×10^{-16}
<i>P4HTM</i>	-0.5187309	2.2×10^{-16}
<i>FSIP1</i>	-0.5091628	2.2×10^{-16}
<i>SIP1</i>	-0.5091628	2.2×10^{-16}
<i>DNAJC12</i>	-0.5090977	2.2×10^{-16}
<i>MLPH</i>	-0.5050419	2.2×10^{-16}
<i>TLE3</i>	-0.4965525	2.2×10^{-16}
Genes correlated to <i>VGLL1</i> expression		
<i>GABRP</i>	0.7576318	2.2×10^{-16}
<i>SLC6A14</i>	0.7236976	2.2×10^{-16}
<i>FOXC1</i>	0.7200038	2.2×10^{-16}
<i>PPP1R14C</i>	0.7152430	2.2×10^{-16}
<i>SFRP1</i>	0.7145003	2.2×10^{-16}
<i>TRIM29</i>	0.7101217	2.2×10^{-16}
<i>PROM1</i>	0.6993393	2.2×10^{-16}
<i>ROM1</i>	0.6993393	2.2×10^{-16}
<i>FABP7</i>	0.6924925	2.2×10^{-16}
<i>BBOX1</i>	0.6924811	2.2×10^{-16}
<i>FOXA1</i>	-0.627766	2.2×10^{-16}
<i>ESR1</i>	-0.625860	2.2×10^{-16}
<i>GATA3</i>	-0.6243054	2.2×10^{-16}
<i>P4HTM</i>	-0.6065218	2.2×10^{-16}
<i>DNAJC12</i>	-0.603839	2.2×10^{-16}
<i>RABEP1</i>	-0.599712	2.2×10^{-16}
<i>TLE3</i>	-0.5975988	2.2×10^{-16}
<i>C6ORF211</i>	-0.5824467	2.2×10^{-16}
<i>SLC16A6</i>	-0.5818284	2.2×10^{-16}
<i>FSIP1</i>	-0.5800131	2.2×10^{-16}

determine a luminal lineage (Perou *et al.* 2000, Sorlie *et al.* 2001), were among the top ten negatively correlated genes (Table 3). The predictions (microRNAs.org database) indicated that miR-934 may interact directly with the regulatory regions of *ESR1*, while there were no indications of direct interactions between this miRNA and the regulatory regions of *FOXA1* and *GATA3*. Hence, the regulation of *FOXA1* and *GATA3* probably occurs indirectly through the inhibition of *ESR1* expression and/or other transcription factors. To obtain further evidence to support this hypothesis, we analyzed two known regulators of *ESR1*: miR-18a and miR-18b (Liu *et al.* 2009; additional file 2, Supplementary Tables 8 and 9, see section on supplementary data given at the end of this article). The similarities between these three miRNAs were clear, and although the correlation and conservation were higher for miR-934 than for both miR-18a and miR-18b, the mirSVR score was slightly lower in the case of miR-934. Indeed, the energy value for miR-934 was between that of miR-18a and miR-18b. These results suggest that miR-934 may directly regulate *ESR1* in a similar way to which it is regulated by miR-18a and miR-18b (additional file 2, Supplementary Table 9). Moreover, protein array data revealed a significant decrease in *ESR1* phosphorylation ($P=7.29 \times 10^{-25}$).

We also investigated the correlation between *VGLL1* and all dataset genes. Interestingly, we found a positive association with luminal progenitor genes (*GABRP*, *SLC6A14*, *FOXC1*, *PROM1*, and *BBOX1*) located between the top ten genes more strongly correlated with *VGLL1* expression. On the other hand, we found a strong negative correlation between *VGLL1* expression and ER-associated genes (*ESR1*, *C6ORF211*, *GATA3*, and *FOXA1*; Table 3).

VGLL1 protein expression in breast carcinomas

We first analyzed the expression pattern of *VGLL1* in positive controls (prostate carcinomas) and normal breast tissues. Nuclear staining was observed in neoplastic epithelial cells in some prostate cancer samples. Nuclear staining was also detected in lymphocytes. In addition, weak cytoplasmic staining was also found in some neoplastic epithelial cells. In normal breast tissue, we did not observe *VGLL1* nuclear expression in either epithelial nor myoepithelial cells. In contrast, we found nuclear expression in some intraepithelial and stromal lymphocytes, detected by CD45 and/or CD3, and/or CD20 immunostaining (additional file 3, Supplementary Fig. 1, see section on supplementary data given at the end of this article). In addition, we analyzed a series of breast carcinoma cell lines representing luminal (MCF7 and

T47D), HER2 (BT474 and SKBR3), and basal subtypes (MCF10, MDA-MB-231, and BT459) by qRT-PCR, and found that *VGLL1* mRNA expression was only detected in SKBR3 and BT474 cell lines (additional file 3, Supplementary Fig. 2). Our results were consistent with those reported by a previous study (Kao *et al.* 2009). These authors observed higher expression of *VGLL1* in basal A breast cancer cell lines, and in some HER2-positive breast cancer cell lines, such as SKBR3, than in basal B breast carcinomas cell lines, such as MCF10, MDA-MB-231, and

Figure 2

(A) Representative immunofluorescence staining of *VGLL1* in the SKBR3 cell line as visualized by immunofluorescence microscopy. Nuclear expression is evident in most cells. In addition, weak cytoplasmic staining (arrow) is also seen in some cells. (B) Representative immunohistochemical nuclear staining of *VGLL1* in a triple-negative invasive breast carcinoma (original magnification, $\times 200$). (C) Immunohistochemical staining of *VGLL1* in a ductal carcinoma *in situ* (original magnification, $\times 200$).

BT459. We carried out immunofluorescence analysis of VGLL1 in MCF-7 (negative control) and SKBR3 (positive control) to evaluate the pattern of protein expression and demonstrated intense nuclear VGLL1 expression and weak cytoplasmic expression in SKBR3 cells (Fig. 2A).

We examined the distribution of the VGLL1 protein in a total of 433 breast carcinomas by IHC carried out on TMA. Given that the only known function of VGLL1 reported to date is a transcriptional co-factor, we only evaluated its nuclear expression (Fig. 2B and C). Tumors were considered positive if nuclear immunoreactivity was evident in >5% of tumor cells.

VGLL1 expression was observed in 46 of 349 invasive breast carcinomas (13.18%) and had no association with patient's age, tumor size, lymph node metastasis, or tumor stage (Table 1). However, significant associations were detected between VGLL1 expression and histological grade, ER, PR, and KI67 expression, as well as with the basal CK (CK5/6 and/or CK17) and c-Kit levels. We found no association between VGLL1 expression and HER2 and EGFR status. Thus, VGLL1 expression was more frequent among grade 3 invasive carcinomas that were ER- and PR-negative, that had a proliferation index $\geq 14\%$ and basal CK expression. Based on these observations, we investigated whether VGLL1 nuclear expression was associated with different molecular phenotypes, as defined in the St Gallen consensus (Goldhirsch et al. 2011). VGLL1 was only occasionally expressed in luminal A (0.70%) and B (5.60%) tumors, but it was expressed in over 40% of TN breast carcinomas (27/67). Tumors that overexpressed HER2 exhibited similar frequencies of VGLL1 expression (17%) regardless of their ER expression. We then investigated whether there was a differential VGLL1 expression between TN breast carcinomas according to their expression of basal markers. Although the frequency of VGLL1 expression appeared to be higher in TN tumors with basal CKs and/or EGFR expression (45.70 vs 34.40%), this difference was not statistically significant.

To confirm these results, we analyzed two additional series, one composed of 70 TN sporadic breast carcinomas (additional file 2, Supplementary Table 2) and another of 14 *BRCA1*-associated carcinomas (including nine TN carcinomas) (additional file 2, Supplementary Table 3). We found that VGLL1 was expressed in 28.5% of sporadic and 55.5% of *BRCA1*-associated TN carcinomas. Considering the three series of invasive breast carcinomas examined in this study, we analyzed a total of 155 TN breast carcinomas, 52 (33.5%) of which expressed VGLL1.

Metaplastic carcinoma is one specific histological type of TN breast carcinomas that expresses basal markers

and that is characterized by the acquisition of an epithelial-to-mesenchymal phenotype (Sarrio et al. 2008, Castilla et al. 2012). Of the TN breast carcinomas analyzed in this study, 16 were metaplastic carcinomas and they all expressed basal CKs and/or EGFR, while 6 (37.5%) expressed VGLL1.

To determine whether VGLL1 was also expressed in the *in situ* component of invasive TN breast carcinomas (Fig. 2C), we analyzed the VGLL1 expression in whole-tissue sections from ten samples (five VGLL1-positive and five VGLL1-negative TN breast carcinomas), in which both *in situ* and invasive components were adjacent in single sections. In all cases, we observed the same profile of VGLL1 expression in both carcinoma components by IHC.

VGLL1 expression and prognosis

A Kaplan–Meier survival analysis of 212 infiltrating breast carcinoma revealed a correlation between VGLL1-positive expression and reduced OS. The follow-up period ranged from 1 to 77 months, and the mean OS was 70.33 months for VGLL1-negative patients and 37.04 months for VGLL1-positive patients ($P=0.0148$; additional file 3, Supplementary Fig. 3, see section on supplementary data given at the end of this article). In univariate Cox proportional hazard, analysis of OS VGLL1 expression was associated with adverse prognosis (Supplementary Table 10). Owing to the limited number of events, we selected the most significant variables for multivariate analysis. The association found between VGLL1 expression and poor prognosis is lost when adjusted for major prognostic factors. There were no significant differences in our cohort of patients on DFS rates between positive VGLL1 expression and negative VGLL1 expression ($P=0.067$; additional file 2, Supplementary Table 10).

Discussion

In this study, we have specifically evaluated the expression of miR-934 and *VGLL1* in breast cancer and for the first time, we demonstrate an association between their expression and a TN-BL phenotype. When we examined the differences in miRNA expression between different groups of highly aggressive breast carcinomas, we identified 28 miRNAs that were differentially expressed between grade 3 ER+/HER2– (comparable to luminal B carcinomas) and grade 3 TN IDCs. These findings were in line with the differential miRNA expression between luminal A and basal-like breast carcinomas found previously (Enerly et al. 2011), where 26 differentially expressed miRNAs

almost perfectly separated the 2 subgroups: 13 upregulated and 13 downregulated in basal-like carcinomas. Among these 26 miRNAs, there were 11 miRNAs (miR-934, miR-9-3p/-5p, miR-135b, miR18a/b, miR-190, miR-149, miR-29c/29c*, and miR-342-3p) that were also differentially expressed in this study, and that may constitute the core miRNA signature of TN breast carcinomas.

The differential expression observed in our study demonstrates that TN breast carcinomas are characterized by the expression of miRNAs that downregulate *ESR1* expression, such as let-7b (Zhao et al. 2011a,b, Quesne et al. 2012) and miR-18a/b (Leivonen et al. 2009, Liu et al. 2009, Yoshimoto et al. 2011). According to this observation, we found that miR-934 was the most significantly modulated miRNA in TN breast cancer, and confirmed this by our analyses of data from the TCGA database (2012). Interestingly, *ESR1* and *C6ORF211* were among the top ten genes most negatively correlated with miR-934 in the TCGA dataset. *C6ORF211* is an open reading frame located immediately upstream of *ESR1* and its expression is strongly correlated with *ESR1* in several publicly available datasets, suggesting transcriptional co-activation (Dunbier et al. 2011). Although the function of miR-934 has not been determined, it is likely to be involved in direct *ESR1* modulation. Our *in silico* comparison between miR-934 and two proven *ESR1* regulators, miR-18a and miR-18b, revealed significant similarities in terms of gene correlations, seed sequence conservation among species, mirSVR scores and energy values. Moreover, among 59 miRNAs that were identified that may target the *ESR1* 3'-UTR (using a luciferase reporter plasmid carrying the entire 3'-UTR of *ESR1*), miR-934 most strongly suppressed the luciferase signal (Xiong et al. 2010).

The significant modulation of miR-934 expression in TN breast carcinomas drew our attention to *VGLL1* gene, as miR-934 is located in intron 4 of *VGLL1*. At present, the role of *VGLL1* in cancer is unknown, although the oncogenic potential of *VGLL1* has been demonstrated in one study reported by Pobbati et al. (2012). As well as a structural and functional similarity between the *VGLL1*-TEAD complex and that formed by TEAD and YAP, it was also demonstrated that *VGLL1*-TEAD complexes promote anchorage-independent cell proliferation. In this study, we first demonstrated that *VGLL1* mRNA was overexpressed in TN breast carcinomas as opposed to ER+ grade 3 IDCs, and that its expression was correlated with that of miR-934, suggesting at least a partial co-regulation.

Through IHC, we demonstrated for the first time that nuclear expression of *VGLL1* protein is associated with TN and basal-like phenotypes. A detailed analysis of several

publicly available datasets revealed that *VGLL1* expression is consistently associated with grade 3 breast carcinomas with a basal-like phenotype (van 't Veer et al. 2002, Farmer et al. 2005, Loi et al. 2007, Lehmann et al. 2011, Moreno-Bueno et al. 2011, Sabatier et al. 2011), confirming our results. Moreover, in the TCGA dataset, *VGLL1* was not only significantly upregulated in basal-like carcinomas with respect to other molecular subtypes but also, it was the gene most strongly correlated with miR-934, supporting the hypothesis of transcriptional coregulation. Although the mechanisms of miR-934/*VGLL1* overexpression are at present unknown, the analysis of TCGA data excluded copy number variation as an important cause.

TN and basal-like breast carcinomas are morphologically and molecularly heterogeneous. A transcriptional analysis of 587 TN carcinomas reported *VGLL1* overexpression in the basal-like 1 carcinoma subtype (Lehmann et al. 2011). This subtype exhibited enhanced expression of cell cycle and DNA damage response genes, and representative cell lines of this group, identified as basal A, also expressed *VGLL1* at higher levels (Kao et al. 2009). Interestingly, these cell lines exhibited an enhanced response to cisplatin and poly ADP ribose polymerase (PARP) inhibitors when compared with cell lines belonging to other TN subtypes.

Despite being a small sample size, our results suggest that *VGLL1* upregulation is more frequent in TN *BRCA1*-associated breast carcinomas. Indeed, *VGLL1* was one of the most strongly upregulated genes in ER-negative *BRCA1*-positive as opposed to ER-negative sporadic carcinomas (van 't Veer et al. 2002). Moreover, cell lines derived from carriers of *BRCA1* mutations are of the basal A/basal 1 subtype (Kao et al. 2009, Lehmann et al. 2011), characterized by *VGLL1* overexpression. *VGLL1* also appears to be a marker of luminal progenitor cells, the cells originating *BRCA1*-associated carcinomas, and a large number of sporadic basal-like carcinomas. Thus, *VGLL1* was one of the genes in a luminal progenitor signature (Lim et al. 2009), along with 5 (*GABRP*, *SLC6A14*, *FOXC1*, *PROM1*, and *BBOX1*) of the ten genes that were most strongly correlated with *VGLL1* in the TCGA dataset. Interestingly, *GABRP*, which was previously associated with basal-like breast carcinomas, was the most strongly correlated with *VGLL1* in the TCGA dataset and it was one of the most strongly upregulated genes in the aforementioned luminal progenitor signature (Lim et al. 2009). Moreover, *PROM1* (CD133), which is also correlated with *VGLL1* in different datasets (Bertheau et al. 2007, Kao et al. 2009), is expressed in a subpopulation of cells

with cancer stem cell properties in *BRCA1*-deficient mouse mammary tumors (Wright *et al.* 2008).

A role of EZH2 and GATA3 in the maintenance of a luminal progenitor phenotype has recently been reported (Granit *et al.* 2012). Indeed, EZH2 was shown to promote a bi-lineage identity in basal-like breast cancer cells (Granit *et al.* 2012), and luminal progenitor-associated genes, including *VGLL1*, were preferentially downregulated in EZH2-silenced cells and upregulated in EZH2-overexpressing cells. By contrast, GATA3, a driver of luminal differentiation, performs an opposite function to that of EZH2, suppressing bi-lineage identity and luminal-progenitor genes expression. Indeed, GATA3 levels increase upon EZH2 silencing, mediating a decrease in bi-lineage cell numbers (Granit *et al.* 2012). Interestingly, the three genes with the greatest negative correlation with *VGLL1* and miR-934 in the TCGA dataset were *GATA3*, *ESR1*, and *FOXA1*, which form a functional enhanceosome that regulates the genes driving core ER α functions and that co-operatively modulates the transcriptional networks previously ascribed to ER α alone (Kong *et al.* 2011). Gain and loss of function *in vitro* studies are currently in progress in our group, using miR-934/*VGLL1*-negative (MCF7 and T47d) and *VGLL1*-positive (SKBR3 and MDA-MB-468) cell lines, respectively, that will confirm the suggested role of miR-934 in the modulation of *ESR1* expression and the maintenance of a luminal progenitor phenotype.

In conclusions, we demonstrate for the first time that *VGLL1*, a transcriptional coactivator with structural similarities to TAZ and YAP, is mainly expressed in a subset of sporadic and *BRCA1*-associated TN basal-like breast carcinomas. An analysis of several publicly available datasets suggests that *VGLL1* is preferentially expressed in tumors and breast cancer cell lines with luminal progenitor features, these exhibiting an enhanced response to cisplatin and PARP inhibitors. Moreover, *VGLL1* expression was correlated with the expression of its intronic miRNA, miR-934, a potential direct modulator of *ESR1* expression. Together, our findings indicate that the coordinated expression of *VGLL1* and miR-934 may be involved in the maintenance of the luminal progenitor phenotype, at least in part mediated by their direct modulation of *ESR1*. These finding might be useful to select specific therapies among TN breast carcinomas.

Future studies should analyze whether or not tumors expressing *VGLL1* might represent a subset of luminal progenitor-enriched breast carcinomas more sensible to specific treatment, such as PARP1 inhibitors and cisplatin.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0485>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by grants from the Instituto de Salud Carlos III (ISCIII, grant numbers PI08/0971 and PI13/02477), co-financed by the European Development Regional Fund ('A way to achieve Europe': grant numbers RD06/0020/0013, RD09/0076/00085, and RD12/0036/0064) and by the Junta de Andalucía (Consejería de Salud, PI0581/2009). M Á Castilla is a PhD researcher funded by the ISCIII (RD06/0020/0013). M R Atienza is a MD researcher funded by the ISCIII (Rio Hortega program). J Daz-Martín and J M Rosa-Rosa are PhD researchers funded by the Consejería de Salud (PI0581/2009) and the ISCIII (RD12/0036/0064) respectively. L Romero-Pérez is a PhD student and a recipient of a PFIS fellowship (grant number F109/00193). M L Pecero works as a lab technician and is supported by the ISCIII (PI080971).

Author contribution statement

M Á Castilla and J Palacios conceived and designed the experiments. J Palacios, M Á López-García, M Á Castilla, M R Atienza, J Benítez, and J Díaz-Martín provided the tumor samples and clinical data. J Palacios, M Á Castilla, M Á López-García, and B Vieites performed the pathological characterization and immunohistochemical samples. M Á Castilla, J M Rosa-Rosa, M L Pecero, L Romero-Pérez, and A Calcabrini carried out the experiments. M Á Castilla, J Palacios, J M Rosa-Rosa, and M Á López-García analyzed and interpreted the data. M Á Castilla and J Palacios wrote the paper with additional input from J M Rosa-Rosa. All authors reviewed the results and discussion.

Acknowledgements

The authors thank Juan Manuel Praena (Statistics, Methodology and Research Evaluation Unit, Andalusian Public Foundation for Health Research Management in Seville) and Javier Zamora (Clinical Biostatistics Unit, Hospital Ramón y Cajal-IRYCIS, Madrid) for help with the statistical analyses.

References

- Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, Fox SB, Ichihara S, Jacquemier J, Lakhani SR *et al.* 2011 Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. *Modern Pathology* **24** 157–167. (doi:10.1038/modpathol.2010.200)
- Bertheau P, Turpin E, Rickman DS, Espie M, de Reynies A, Feugeas JP, Plassa LF, Soliman H, Varna M, de Roquancourt A *et al.* 2007 Exquisite sensitivity of TP53 mutant and basal breast cancers to a dose-dense epirubicin-cyclophosphamide regimen. *PLoS Medicine* **4** e90. (doi:10.1371/journal.pmed.0040090)
- Castilla MA, Moreno-Bueno G, Romero-Perez L, Van De Vijver K, Biscuola M, Lopez-Garcia MA, Prat J, Matias-Guiu X, Cano A, Oliva E *et al.* 2011 Micro-RNA signature of the epithelial-mesenchymal transition in endometrial carcinosarcoma. *Journal of Pathology* **223** 72–80. (doi:10.1002/path.2802)

- Castilla MA, Diaz-Martin J, Sarrio D, Romero-Perez L, Lopez-Garcia MA, Vieites B, Biscuola M, Ramiro-Fuentes S, Isacke CM & Palacios J 2012 MicroRNA-200 family modulation in distinct breast cancer phenotypes. *PLoS ONE* **7** e47709. (doi:10.1371/journal.pone.0047709)
- Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E et al. 2012 The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discovery* **2** 401–404. (doi:10.1158/2159-8290.CD-12-0095)
- Dunbier AK, Anderson H, Ghazoui Z, Lopez-Knowles E, Pancholi S, Ribas R, Drury S, Sidhu K, Leary A, Martin LA et al. 2011 ESR1 is co-expressed with closely adjacent uncharacterised genes spanning a breast cancer susceptibility locus at 6q25.1. *PLoS Genetics* **7** e1001382. (doi:10.1371/journal.pgen.1001382)
- Dweep H, Sticht C, Pandey P & Gretz N 2011 miRWalk – database: prediction of possible miRNA binding sites by ‘walking’ the genes of three genomes. *Journal of Biomedical Informatics* **44** 839–847. (doi:10.1016/j.jbi.2011.05.002)
- Enerly E, Steinfeld I, Kleivi K, Leivonen SK, Aure MR, Russnes HG, Ronneberg JA, Johnsen H, Navon R, Rodland E et al. 2011 miRNA-mRNA integrated analysis reveals roles for miRNAs in primary breast tumors. *PLoS ONE* **6** e16915. (doi:10.1371/journal.pone.0016915)
- Farmer P, Bonnefoi H, Becette V, Tubiana-Hulin M, Fumoleau P, Larsimont D, Macgrogan G, Bergh J, Cameron D, Goldstein D et al. 2005 Identification of molecular apocrine breast tumours by microarray analysis. *Oncogene* **24** 4660–4671. (doi:10.1038/sj.onc.1208561)
- Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B & Senn HJ 2011 Strategies for subtypes – dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. *Annals of Oncology* **22** 1736–1747. (doi:10.1093/annonc/mdr304)
- Granit RZ, Gabai Y, Hadar T, Karamansha Y, Liberman L, Waldhorn I, Gat-Viks I, Regev A, Maly B, Darash-Yahana M et al. 2012 EZH2 promotes a bi-lineage identity in basal-like breast cancer cells. *Oncogene* **32** 3886–3895. (doi:10.1038/ncr.2012.390)
- Kao J, Salari K, Bocanegra M, Choi YL, Girard L, Gandhi J, Kwei KA, Hernandez-Boussard T, Wang P, Gazdar AF et al. 2009 Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PLoS ONE* **4** e6146. (doi:10.1371/journal.pone.0006146)
- Kong SL, Li G, Loh SL, Sung WK & Liu ET 2011 Cellular reprogramming by the conjoint action of E α , FOXA1, and GATA3 to a ligand-inducible growth state. *Molecular Systems Biology* **7** 526. (doi:10.1038/msb.2011.59)
- Lamar JM, Stern P, Liu H, Schindler JW, Jiang ZG & Hynes RO 2012 The Hippo pathway target, YAP, promotes metastasis through its TEAD-interaction domain. *PNAS* **109** E2441–E2450. (doi:10.1073/pnas.1212021109)
- Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y & Pietenpol JA 2011 Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *Journal of Clinical Investigation* **121** 2750–2767. (doi:10.1172/JCI45014)
- Leivonen SK, Makela R, Ostling P, Kohonen P, Haapa-Paananen S, Kleivi K, Enerly E, Aakula A, Hellstrom K, Sahlberg N et al. 2009 Protein lysate microarray analysis to identify microRNAs regulating estrogen receptor signaling in breast cancer cell lines. *Oncogene* **28** 3926–3936. (doi:10.1038/ncr.2009.241)
- Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, Asselin-Labat ML, Gyorki DE, Ward T, Partanen A et al. 2009 Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nature Medicine* **15** 907–913. (doi:10.1038/nm.2000)
- Liu WH, Yeh SH, Lu CC, Yu SL, Chen HY, Lin CY, Chen DS & Chen PJ 2009 MicroRNA-18a prevents estrogen receptor- α expression, promoting proliferation of hepatocellular carcinoma cells. *Gastroenterology* **136** 683–693. (doi:10.1053/j.gastro.2009.02.016)
- Loi S, Haibe-Kains B, Desmedt C, Lallemand F, Tutt AM, Gillet C, Ellis P, Harris A, Bergh J, Foekens JA et al. 2007 Definition of clinically distinct molecular subtypes in estrogen receptor-positive breast carcinomas through genomic grade. *Journal of Clinical Oncology* **25** 1239–1246. (doi:10.1200/JCO.2006.07.1522)
- Moreno-Bueno G, Salvador F, Martin A, Floristan A, Cuevas EP, Santos V, Montes A, Morales S, Castilla MA, Rojo-Sebastian A et al. 2011 Lysyl oxidase-like 2 (LOXL2), a new regulator of cell polarity required for metastatic dissemination of basal-like breast carcinomas. *EMBO Molecular Medicine* **3** 528–544. (doi:10.1002/emmm.201100156)
- Palacios J, Honrado E, Osorio A, Cazorla A, Sarrio D, Barroso A, Rodriguez S, Cigudosa JC, Diez O, Alonso C et al. 2005 Phenotypic characterization of BRCA1 and BRCA2 tumors based in a tissue microarray study with 37 immunohistochemical markers. *Breast Cancer Research and Treatment* **90** 5–14. (doi:10.1007/s10549-004-1536-REF21=10.1007/s10549-004-1536-0)
- Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA et al. 2000 Molecular portraits of human breast tumours. *Nature* **406** 747–752. (doi:10.1038/35021093)
- Pobbati AV, Chan SW, Lee I, Song H & Hong W 2012 Structural and functional similarity between the Vgll1-TEAD and the YAP-TEAD complexes. *Structure* **20** 1135–1140. (doi:10.1016/j.str.2012.04.004)
- Quesne JL, Jones J, Warren J, Dawson SJ, Ali HR, Bardwell H, Blows F, Pharoah P & Caldas C 2012 Biological and prognostic associations of miR-205 and let-7b in breast cancer revealed by *in situ* hybridization analysis of micro-RNA expression in arrays of archival tumour tissue. *Journal of Pathology* **227** 306–314. (doi:10.1002/path.3983)
- Sabatier R, Finetti P, Cervera N, Lambaudie E, Esterni B, Mamessier E, Tallet A, Chabannon C, Extra JM, Jacquemier J et al. 2011 A gene expression signature identifies two prognostic subgroups of basal breast cancer. *Breast Cancer Research and Treatment* **126** 407–420. (doi:10.1007/s10549-010-0897-9)
- Sarrio D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G & Palacios J 2008 Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer Research* **68** 989–997. (doi:10.1158/0008-5472.CAN-07-2017)
- Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS et al. 2001 Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *PNAS* **98** 10869–10874. (doi:10.1073/pnas.191367098)
- Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S et al. 2003 Repeated observation of breast tumor subtypes in independent gene expression data sets. *PNAS* **100** 8418–8423. (doi:10.1073/pnas.0932692100)
- The Cancer Genome Atlas Network 2012 Comprehensive molecular portraits of human breast tumours. *Nature* **490** 61–70. (doi:10.1038/nature11412)
- Toft DJ & Cryns VL 2011 Minireview: Basal-like breast cancer: from molecular profiles to targeted therapies. *Molecular Endocrinology* **25** 199–211. (doi:10.1210/me.2010-0164)
- Vaudin P, Delanoue R, Davidson I, Silber J & Zider A 1999 TONDU (TDU), a novel human protein related to the product of vestigial (vg) gene of *Drosophila melanogaster* interacts with vertebrate TEF factors and substitutes for Vg function in wing formation. *Development* **126** 4807–4816.
- van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT et al. 2002 Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415** 530–536. (doi:10.1038/415530a)
- Wright MH, Calcagno AM, Salcido CD, Carlson MD, Ambudkar SV & Varticovski L 2008 Brca1 breast tumors contain distinct CD44+/CD24– and CD133+ cells with cancer stem cell characteristics. *Breast Cancer Research* **10** R10. (doi:10.1186/bcr1855)
- Xiong J, Yu D, Wei N, Fu H, Cai T, Huang Y, Wu C, Zheng X, Du Q, Lin D et al. 2010 An estrogen receptor α suppressor, microRNA-22, is downregulated in estrogen receptor α -positive human breast cancer cell

- lines and clinical samples. *FEBS Journal* **277** 1684–1694. (doi:10.1111/j.1742-4658.2010.07594.x)
- Yoshimoto N, Toyama T, Takahashi S, Sugiura H, Endo Y, Iwasa M, Fujii Y & Yamashita H 2011 Distinct expressions of microRNAs that directly target estrogen receptor α in human breast cancer. *Breast Cancer Research and Treatment* **130** 331–339. (doi:10.1007/s10549-011-1672-2)
- Zecca M & Struhl G 2010 A feed-forward circuit linking wingless, fat-dachsous signaling, and the Warts–Hippo pathway to *Drosophila* wing growth. *PLoS Biology* **8** e1000386. (doi:10.1371/journal.pbio.1000386)
- Zhao Y, Deng C, Lu W, Xiao J, Ma D, Guo M, Recker RR, Gatalica Z, Wang Z & Xiao GG 2011a let-7 microRNAs induce tamoxifen sensitivity by downregulation of estrogen receptor α signaling in breast cancer. *Molecular Medicine* **17** 1233–1241. (doi:10.2119/molmed.2010.00225)
- Zhao Y, Deng C, Wang J, Xiao J, Gatalica Z, Recker RR & Xiao GG 2011b Let-7 family miRNAs regulate estrogen receptor α signaling in estrogen receptor positive breast cancer. *Breast Cancer Research and Treatment* **127** 69–80. (doi:10.1007/s10549-010-0972-2)

Received in final form 27 May 2014

Accepted 29 May 2014

Made available online as an Accepted Preprint

2 June 2014