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RESEARCH ARTICLE

REVISED Spatial intratumoural heterogeneity in the expression of GIT1 is associated with poor prognostic outcome in oestrogen receptor positive breast cancer patients with synchronous lymph node metastases [version 2; referees: 2 approved with reservations]

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Abstract

Background: The outcome for oestrogen receptor positive (ER+) breast cancer patients has improved greatly in recent years largely due to targeted therapy. However, the presence of involved multiple synchronous lymph nodes remains associated with a poor outcome. Consequently, these patients would benefit from the identification of new prognostic biomarkers and therapeutic targets. The expression of G-protein-coupled receptor kinase-interacting protein 1 (GIT1) has recently been shown to be an indicator of advanced stage breast cancer. Therefore, we investigated its expression and prognostic value of GIT1 in a cohort of 140 ER+ breast cancer with synchronous lymph node involvement.
Methods: Immunohistochemistry was employed to assess GIT1 expression in a tissue microarray (TMA) containing duplicate non-adjacent cores with matched primary tumour and lymph node tissue (n=140). GIT1 expression in tumour cells was scored and statistical correlation analyses were carried out.
Results: The results revealed a sub-group of patients that displayed discordant expression of GIT1 between the primary tumour and the lymph nodes (i.e. spatial intratumoural heterogeneity). We observed that loss of GIT1 expression

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version 1 published 30 Aug 2017	? report	? report

1 **Mona M. Mohamed**, Cairo University, Egypt

in the tumour cells of the metastasis was associated with a shorter time to recurrence, poorer overall survival, and a shorter median survival time. Moreover, multivariate analysis demonstrated that GIT1 expression was an independent prognostic indicator.
Conclusions: GIT1 expression enabled the identification of a sub-class of ER+ patients with lymph node metastasis that have a particularly poor prognostic outcome. We propose that this biomarker could be used to further stratify ER+ breast cancer patients with synchronous lymph node involvement and therefore facilitate adjuvant therapy decision making.

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REVISED **Amendments from Version 1**

We would like to thank the insightful comments made by reviewers which further enriched the revised manuscript. I hope we are able to satisfy most of your concerns regarding the manuscript. Figure 1 and Supplementary Figure S2 have been updated, as scale bars have been included on them. In addition, Dataset 1–Dataset 8 have been uploaded in CSV format, as suggested by reviewers; no other changes have been made to the data itself. 3 new references had been added to Discussion.

See referee reports

Introduction

Breast cancer is the most common type of cancer among Western women, and every year around 450,000 new cases are diagnosed in Europe alone[1]. Breast cancer is recognised as a very heterogeneous disease and several different subgroups have been identified on the basis of hormone receptor and Her2 status, or more recently by gene expression profiling[2]. Each subgroup shows different pathological, clinical and molecular characteristics, which in turn have different therapeutic options and prognostic outcome. The oestrogen-positive (ER+) breast cancer subtype is the most common of these subtypes representing around 70% of all cases. Although the majority of ER+ cases respond to anti-oestrogen treatments, some are resistant to endocrine therapies, in particular those patients with involved lymph nodes and distant metastases at time of presentation[3,4]. Indeed, the presence of synchronous lymph node metastasis is a strong indicator of poor prognostic outcome[5]. Therefore, there is a clear clinical need to identify new therapeutic targets and biomarkers for this group of patients including those that do not relapse.

It has been shown recently that advanced stage breast cancer and involved lymph nodes express high mRNA levels of G-protein-coupled receptor kinase-interacting protein 1 (GIT1)[6], however, this study did not examine GIT1 protein expression, with the exception of nine tumours. As immunohistochemistry (IHC) exhibits greater potential for clinical application we decided to evaluate GIT1 immunoreactivity in an extensive cohort of ER+ breast cancer cases with synchronous lymph node (LN) involvement (n=140). We evaluated the association of GIT1 expression in the primary tumour and in the synchronous LN with clinical features and disease aggressiveness.

Methods

Patient cohort

Primary site and lymph node metastasis tissue samples were obtained from the Pathology Department of Donostia University Hospital from 140 breast patients who were diagnosed between 2000 and 2006, with follow-up data until 2014 with a median follow-up of 8 years and 8 months. Written consent was obtained from patients for the inclusion of their samples in this study and samples were collected in accordance with the Declaration of Helsinki, and approved by local ethics committees (Comite Etico de Investigación Clínica de Euskadi (CEIC-E)). Cases were selected and re-reviewed by two experienced breast pathologists independently. Clinical data was only available for 104 of these patients (Dataset 1). All patients were women with an age range between 27 and 86 years old (median age 58 years old). All primary site tumours were ER+ and 91% of them PR+. Eighty-six percent of patients presented with invasive carcinoma of no special type (NST) and 11% with invasive lobular carcinoma (ILC). Histological grades varied from grade I (21%), grade II (55%) to grade III (15%), according to the Elston-Ellis modification of Scarff-Bloom-Richardson grading system. All patients were surgically treated either by tumorectomy or by mastectomy with axillary lymphadenectomy. All patients underwent hormone therapy with the majority of them receiving radiotherapy (90%) and adjuvant chemotherapy (75%). Table 1 summarizes the clinical and histological characteristics of patients as we previously described[7].

Tissue microarray construction and immunohistochemistry (IHC)

Representative areas of high tumour load (>70%) were selected after H&E staining and two 1.5mm punch biopsies taken from both primary tumour and lymph node and arrayed non-adjacently to reduce staining bias using a Manual Tissue Arrayer MTA-1 (Beecher Instruments, USA).

Immunohistochemical (IHC) staining was carried out on 5µm slices manually using the Immunohistochemistry Accessory Kit of Bethyl Laboratories (Montgomery, USA). Slides were deparaffinized in xylene and blocked in peroxidase for 30 min. Antigen retrieval was carried out in Epitope Retrieval Buffer (Bethyl Laboratories, Montgomery, USA). Slides were blocked in BSA for 30 min and then incubated for 1h at room temperature with anti-GIT1 rabbit polyclonal antibody (1:100) (IHC-00527 (lot #001), Bethyl Laboratories, Montgomery, USA). Even though this is the same antibody used by the study of Chan *et al.*[6], and raised against a GIT1-specific peptide immunogen, we cannot rule out cross-reactivity against other non-GIT1 epitopes. After 1h incubation with secondary anti-Rabbit IHC Antibody and DAB substrate. The slides counterstained with hematoxylin.

GIT1 expression was scored in a blinded fashion by an experienced breast pathologist according to tumour cell staining intensity and categorical scores assigned as follows; 0= negative (0%); 1=1–10%; 2= 11–50%; 3= >50% (Dataset 2). Scores between non-adjacent cores were combined and categorised according to following criteria; GIT1 negative (combined score <1); moderate GIT1 expression (combined score 1–2); and high GIT1 expression (score >2). Examples of the different staining categories are shown in Figure 1 and Supplementary Figure S2. A selection of cases (n=8) were examined both as whole biopsy sections and TMAs, all scoring was concordant. Examples of whole section IHC staining with GIT1 are shown in Supplementary Figure S1 and Supplementary Figure S3.

Dataset 1. Clinical data of patient cohort

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Table shows patients (numbered from 1 to 105) and their clinical features including histological subgroup, tumour size, number of affected lymph nodes, histological grade, vascular lymphatic infiltration, immunohistochemical initial status, treatment and patient follow up.

Table 1. Clinical and histological characteristics of patients used in study.

Features			n (%)
Histological subgroup	NSC		90 (86.5)
	ILC		12 (11.5)
	other		2 (1.9)
Tumour size (mm)	<20		40 (38.8)
	20–40		53 (51.5)
	>40		10 (9.7)
Number of affected lymph nodes	<5		70 (68.0)
	5–10		24 (23.3)
	>10		9 (8.7)
Histological grade	I		22 (21.4)
	II		57 (55.3)
	III		15 (14.6)
	Unknown		9 (8.7)
Vascular lymphatic infiltration	No		81 (78.6)
	Yes		22 (21.4)
Immunohistochemical initial status	ER	Positive	103 (100.0)
		Negative	0 (0.0)
	PR	Positive	94 (91.3)
		Negative	9 (8.7)
	HER2	Score 0	46 (44.7)
		Score 1	43 (41.7)
		Score 2	7 (6.8)
		Score 3	7 (6.8)
Treatment	Surgery	Tumorectomy	55 (53.4)
		Mastectomy	49 (47.6)
	Radiotherapy	No	8 (7.8)
		Breast conserving therapy	55 (53.4)
		Thoracic wall	39 (37.9)
	Adjuvant chemotherapy	NO	25 (24.3)
		YES	78 (75.7)
	Hormone therapy	NO	1 (1.0)
		Tamoxifen	34 (33.0)
		AI	25 (24.3)
		Tmx -> AI	42 (40.8)
Follow up	Recurrence	NO	77 (74.8)
		YES	27 (26.2)
	Distant metastasis	NO	73 (70.9)
		YES	31 (30.1)
	Death	NO	69 (67.0)
		YES	36 (35.0)

[i] Abbreviations: n (%) = number of patients (percentage within each feature), AI = Aromatase inhibitors, Tmx -> AI = Tamoxifen followed by Aromatase inhibitors. NST, invasive carcinoma of no special type; ILC, invasive lobular carcinoma. p values calculated with Chi-square contingency test.

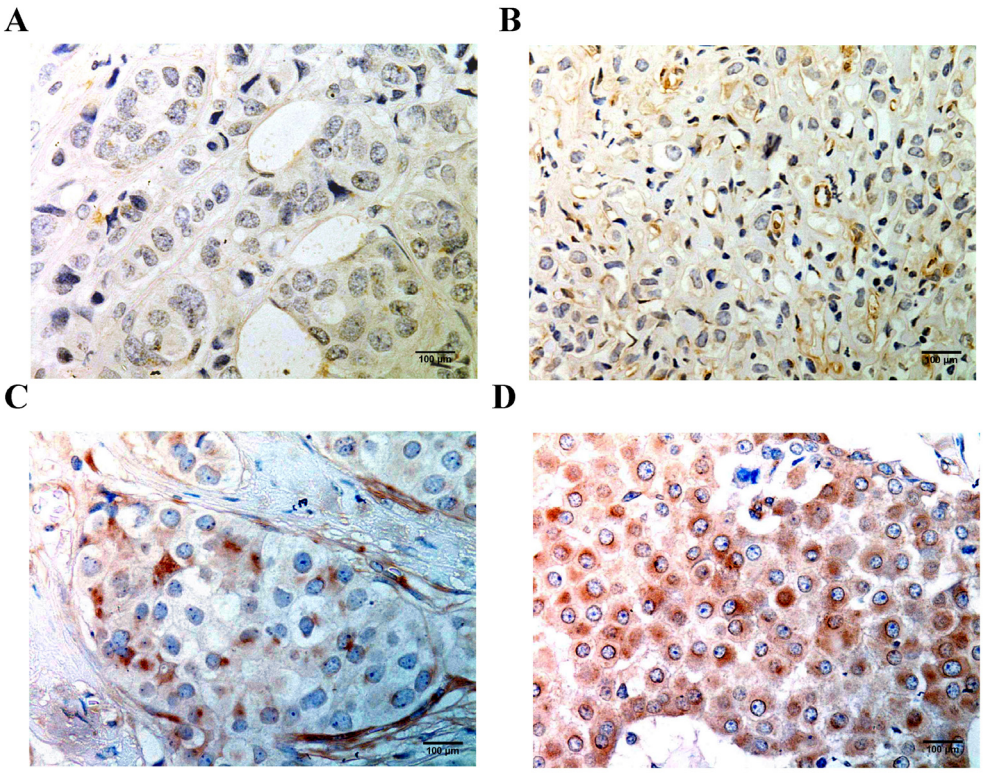

Figure 1. Example of GIT1 expression patterns found in ER+ breast cancer. Images were enhanced from the original (Supplementary Figure S2). Representative intensity staining of GIT1 expression (primary tumour) depicting **A**. negative (score=0); **B**. weak (score=1); **C.** moderate (score=2); and **D**. strong (score=3). Magnification ×400.

Dataset 2. GIT1 scoring

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Table shows patients (numbered from 1 to 142) and associated primary tumour and lymph node GIT1 scoring. Categorical scores are assigned as follows according to tumour cell staining intensity; 0= negative (0%); 1=1–10%; 2= 11–50%; 3= >50%.

Dataset 3. Series mRNA expression matrix and clinical data information

http://dx.doi.org/10.5256/f1000research.12393.d194224

GIT1 Expression Dataset consisting of 522 primary tumors, 3 metastatic tumors, and 22 tumor-adjacent normal samples. Data was median centered by genes. Platform: Affymetrix Human Genome U133A Array. Publicly available from https://tcga-data.nci.nih.gov/docs/publications/brca_2012/.

Dataset 4. Series mRNA expression matrix

http://dx.doi.org/10.5256/f1000research.12393.d194225

Expression Dataset consisting of 2000 breast carcinoma. Platform: Affymetrix Human HT-12 V3 Array. Publicly available from http://www.cbioportal.org/study?id=brca_metabric#summary

Dataset 5. Series mRNA expression matrix

http://dx.doi.org/10.5256/f1000research.12393.d194226

Expression Dataset consisting of one hundred fifty-four (154) invasive breast carcinoma samples and 4 normal breast samples. Platform: Agilent UNC Perou Lab Homo sapiens 1X44K Custom Array. Publicly available from Gluck Breast dataset (https://www.oncomine.org)

Dataset 6. Series mRNA expression matrix

http://dx.doi.org/10.5256/f1000research.12393.d194227

Expression Dataset consisting of 252 lymph-node negative breast cancer samples. Platform: Affymetrix Human Genome U133A Array. Publicly available from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=gse2034

Dataset 7. Series mRNA expression matrix

http://dx.doi.org/10.5256/f1000research.12393.d194228

Expression Dataset consisting of 67 triple negative breast cancer samples. Platform: Affymetrix Human Genome U133A Array. Publicly available from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31519

Dataset 8. Series mRNA expression matrix

http://dx.doi.org/10.5256/f1000research.12393.d194229

Expression Dataset consisting of 19 HER2+ brain metastasis breast cancer samples and 19 HER2+ non-metastatic breast cancer samples. Platform: Affymetrix Human X3P Array. Publicly available from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43837

Public dataset analysis

GIT1 gene expression levels were analyzed using publicly available databases. For this analyses we interrogated the TCGA dataset which included 525 mixed breast cancer tumours and 22 normal breast samples (Dataset 3)[8], 2000 mixed breast tumours (Dataset 4)[9], 570 metastatic breast tumours (Dataset 5)[10], 252 lymph-node negative breast cancer patients (Dataset 6)[11], 67 triple negative breast cancer patients (Dataset 7)[12], and 19 primary breast cancer and 19 brain metastasis from HER2 positive breast cancer patients (Dataset 8)[13].

Statistical analysis

Chi-square statistical test was used to determine association between GIT1 expression and lymph node metastasis (Table 3). Fisher's exact test and Chi-square test were used to associate GIT1 expression with clinicopathological features (Table 1, Table 2 and Table 4). For survival analysis, Kaplan-Meier curves

Table 2. Association between GIT1 expression levels and clinicopathological features.

Features			GIT1 expression, n (%)			p value
			Negative	Moderate	High	
Histological subgroup	Ductal		22 (84.6)	34 (91.9)	34 (82.9)	0.6436
	Lobular		3 (11.5)	3 (8.1)	6 (14.6)	
	other		1 (3.8)	0 (0.0)	1 (2.4)	
Tumour size (mm)	<20		10 (38.5)	16 (44.4)	14 (34.1)	0.6732
	20–40		14 (53.8)	18 (50.0)	21 (51.2)	
	>40		2 (7.7)	2 (5.6)	6 (14.6)	
Number of affected lymph nodes	>5		18 (69.2)	25 (69.4)	27 (65.9)	0.823
	5–10		7 (26.9)	8 (22.2)	9 (22.0)	
	>10		1 (3.8)	3 (8.3)	5 (12.2)	
Histological grade	I		8 (30.8)	5 (13.9)	9 (22.0)	0.5075
	II		13 (50.0)	21 (58.3)	23 (56.1)	
	III		4 (15.4)	7 (19.4)	4 (9.8)	
	Unknown		1 (3.8)	3 (8.3)	5 (12.2)	
Vascular lymphatic infiltration	No		21 (80.8)	27 (75.0)	33 (80.5)	0.8035
	Yes		5 (19.2)	9 (25.0)	8 (19.5)	
Immunohistochemical initial status	ER	Positive	26 (100.0)	36 (100.0)	41 (100.0)	n.a.
		Negative	0 (0.0)	0 (0.0)	0 (0.0)	
	PR	Positive	24 (92.3)	34 (94.4)	36 (87.8)	0.5748
		Negative	2 (7.7)	2 (5.6)	5 (12.2)	
	HER2	Score 0	12 (46.2)	16 (44.4)	18 (43.9)	0.4251
		Score 1	9 (34.6)	15 (41.7)	19 (46.3)	
		Score 2	4 (15.4)	1 (2.8)	2 (4.9)	
		Score 3	1 (3.8)	4 (11.1)	2 (4.9)	
Treatment	Surgery	Tumorectomy	15 (57.7)	17 (47.2)	23 (56.1)	0.649
		Mastectomy	11 (42.3)	19 (52.8)	18 (43.9)	
	Radiotherapy	No	0 (0.0)	4 (11.1)	4 (9.8)	0.3749
		Breast conserving therapy	15 (57.7)	16 (44.4)	24 (58.5)	
		Thoracic wall	11 (42.3)	15 (41.7)	13 (31.7)	
	Adjuvant chemotherapy	NO	6 (23.1)	9 (25.0)	10 (24.4)	0.9721
		YES	20 (76.9)	26 (72.2)	31 (75.6)	
	Hormone therapy	NO	0 (0.0)	1 (2.8)	0 (0.0)	0.1941
		Tamoxifen	9 (34.6)	14 (38.9)	11 (26.8)	
		AI	9 (34.6)	9 (25.0)	7 (17.1)	
		Tmx -> AI	8 (30.8)	11 (30.6)	23 (56.1)	

[I] Abbreviations: n (%) = number of patients (percentage within each feature), AI = Aromatase inhibitors, Tmx -> AI = Tamoxifen followed by Aromatase inhibitors. p values calculated with Chi-square contingency test

Table 3. Relationship between GIT1 expression in primary tumour and lymph node metastasis.

	GIT1 expression, n (%)			p value
	Negative	Moderate	High	
Tumour	35 (25.0)	58 (41.4)	47 (33.6)	0.0054
Lymph node metastasis	60 (42.9)	40 (28.6)	40 (28.6)	

[i] Abbreviations: n (%) = number of patients (percentage with respect to all patients "140"). p value calculated with Chi-square contingency test.

Table 4. Comparison of GIT1 +/- patients and the rest of the patients with clinicopathological features.

Features			GIT1 expression, n (%)		p value
			+/- Group	Rest of patients	
Histological subgroup	Ductal		27 (87.1)	62 (87.3)	>0.9999
	Lobular		3 (9.7)	8 (11.3)	
	other		1 (3.2)	1 (1.4)	
Tumour size (mm)	<20		11 (35.5)	29 (40.8)	0.7416
	20–40		16 (51.6)	36 (50.7)	
	>40		4 (12.9)	6 (8.5)	
Number of affected lymph nodes	<5		23 (74.2)	46 (64.8)	0.6389
	5–10		6 (19.4)	18 (25.4)	
	>10		2 (6.5)	7 (9.9)	
Histological grade	I		5 (16.1)	16 (22.5)	0.3235
	II		17 (54.8)	40 (56.3)	
	III		7 (22.6)	8 (11.3)	
	Unknown		2 (6.5)	7 (9.9)	
Vascular lymphatic infiltration	No		22 (73.1)	58 (81.7)	0.2954
	Yes		9 (26.9)	13 (18.3)	
Immunohistochemical initial status	ER	Positive	31 (100.0)	71 (100.0)	n.a.
		Negative	0 (0.0)	0 (0.0)	
	PR	Positive	30 (96.8)	63 (88.7)	0.2701
		Negative	1 (3.2)	8 (11.3)	
	HER2	Score 0	14 (45.2)	31 (43.7)	0.2985
		Score 1	14 (45.2)	29 (40.8)	
		Score 2	0 (0.0)	7 (9.9)	
		Score 3	3 (9.7)	4 (5.6)	
Treatment	Surgery	Tumorectomy	14 (45.2)	41 (57.7)	0.2836
		Mastectomy	17 (54.8)	30 (42.3)	
	Radiotherapy	No	3 (9.7)	5 (7.0)	0.4257
		Breast conserving therapy	14 (45.2)	42 (59.2)	
		Thoracic wall	14 (45.2)	24 (33.8)	
	Adjuvant chemotherapy	NO	6 (19.4)	19 (26.8)	0.466
		YES	25 (80.6)	52 (73.2)	
	Hormone therapy	NO	0 (0.0)	1 (1.4)	0.4376
		Tamoxifen	13 (41.9)	21 (29.6)	
		AI	5 (16.1)	20 (28.2)	
		Tmx -> AI	13 (41.9)	28 (39.4)	

[i] Abbreviations: n (%) = number of patients (percentage within each feature), AI = Aromatase inhibitors, Tmx -> AI = Tamoxifen followed by Aromatase inhibitors. p values calculated with Chi-square contingency test

and univariate Log-rank (Mantel-Cox) analysis were performed (Figure 2 and Figure 3E). Statistical analyses were performed using GraphPad Prism v5.03 (GraphPad Software, La Jolla, CA, United States). Cox regression for multivariate analysis was performed with SPSS Statistics 20 (IBM, New York, USA) (Table 5).

For public dataset analysis, expression data were analyzed by t-test when comparing 2 groups or Anova when comparing more than 2 groups (Figure 3). Data was analyzed using GraphPad Prism v5.03 (CA, United States) and R (R Foundation for Statistical Computing, Vienna, Austria).

Results

Analysis of GIT1 expression in primary tumours

We observed both cytoplasmic and membrane expression of GIT1 in tumour cells of varying intensities, along with some perinuclear localisation and some weaker signal in stromal cells (Figure 1). This staining pattern is consistent with that previously observed[6,14].

In order to ascertain the association of tumoural GIT1 expression with clinicopathological characteristics, we scored the expression according to intensity and % positive tumour cells in each case (based on two cores) as negative, moderate and high (Figure 1). Out of the 140 primary tumour specimens, we observed high expression of GIT1 in 47 cases (34%), moderate expression in 58 cases (42%) and no GIT1 expression in 35 cases (25%). There was no significant association between GIT1 expression and the 2012 WHO defined histological subtype (i.e. invasive carcinoma of no special type (NST; n=90 (86.5%)), invasive lobular carcinoma (ILC; n=12 (11.5%)) or other). Neither were there significant associations with hormone receptor status, tumour size, number of affected lymph nodes, histological grade or the presence of vascular lymphatic infiltrate (Table 2). We observed no significant difference in the overall survival (OS) (Figure 2A), or time to recurrence in patients according to the level of GIT1 expression in these primary tumours (data not shown).

Spatial intratumoural heterogeneity of GIT1 expression in ER+ breast cancer

When we compared GIT1 expression in primary tumours with that of their counterpart lymph node metastases surprisingly we observed a significant decrease of GIT1 expression (p=0.0054, Table 3). Although both lymph node and primary biopsies showed a similar frequency of high GIT1 expression (29% vs 34%

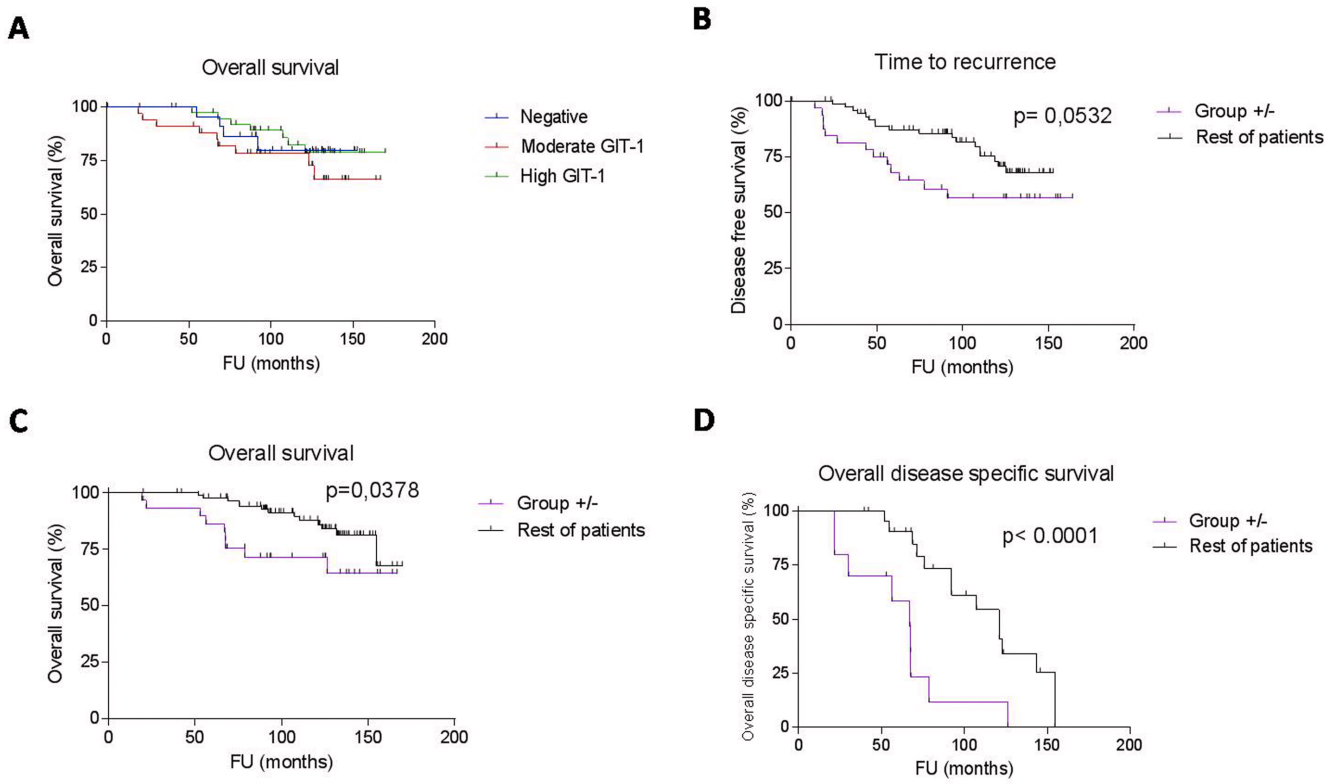
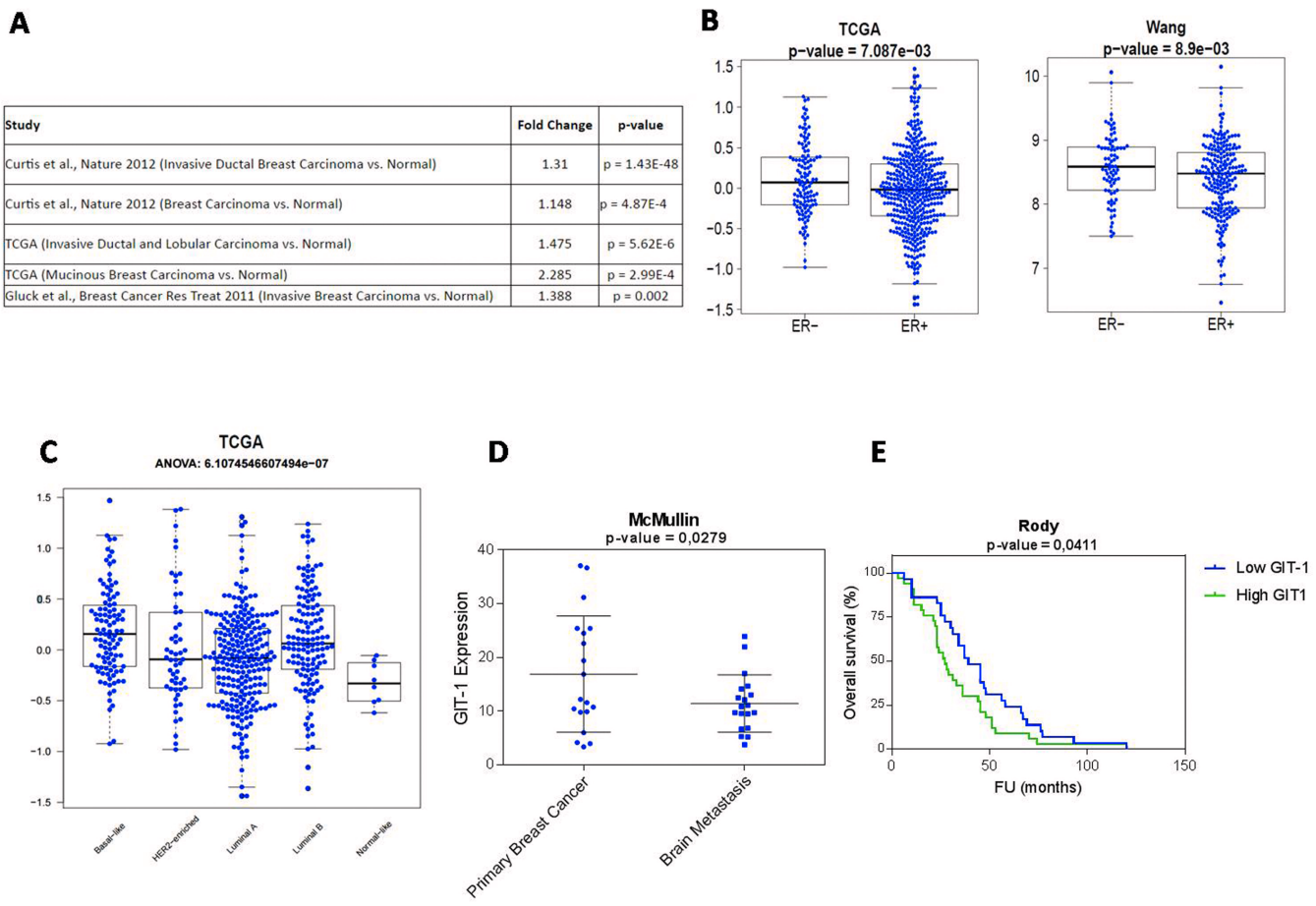


Figure 2. Kaplan-Meier survival curves of ER+/LN+ breast cancer cases according to GIT1 expression. Curves were compared by univariate (log-rank) analysis. **A**. Cases sub-classified according to expression levels of GIT1 in the primary tumour (or LN) were not significantly different. Cases that were GIT1 +/- (n=31) had a shorter time to recurrence (**B**) and overall survival (**C**), and disease specific survival (**D**) than non GIT1+/- cases (n=73).



Study	Fold Change	p-value
Curtis et al., Nature 2012 (Invasive Ductal Breast Carcinoma vs. Normal)	1.31	p = 1.43E-48
Curtis et al., Nature 2012 (Breast Carcinoma vs. Normal)	1.148	p = 4.87E-4
TCGA (Invasive Ductal and Lobular Carcinoma vs. Normal)	1.475	p = 5.62E-6
TCGA (Mucinous Breast Carcinoma vs. Normal)	2.285	p = 2.99E-4
Gluck et al., Breast Cancer Res Treat 2011 (Invasive Breast Carcinoma vs. Normal)	1.388	p = 0.002

Figure 3. GIT1 expression in different breast cancer subtypes. A. GIT1 expression is significantly higher in breast cancer samples compared to normal breast cancer tissue. *In silico* meta-analysis of five databases representing 3452 cases. **B**. In two independent datasets GIT1 expression was lower in ER positive compared to ER negative tumours. **C**. GIT1 expression is highest in the basal breast cancer subtype which represents ER-cases (ANOVA analysis). **D**. GIT1 expression is lower in brain metastasis than primary breast tumour sites. **E**. Survival analysis of triple negative breast cancer patients show low GIT1 expression is associated with poorer clinical outcome. Patients were sub-classified on the basis of median GIT1 expression levels (univariate log rank test). Unless otherwise specified analysis was carried out by independent *t*-test.

Table 5. Cox regression multivariate analysis of overall survival in patients with breast cancer.

	P value	Hazard ratio	95% CI
Distant metastasis	0.943	>10	0 - >10
Histological grade	0.127	1.811	0.84 – 3.88
Group +/-	0.045	2.759	1.02 – 7.44

[i] Table shows the relevance of presence of distant metástasis, a high histological grade (III) and being in the group +/- as predictor variables to overall survival. p values are calculated with Cox regression for multivariate analysis test.

respectively), the percentage of lymph nodes with moderate GIT1 expression was lower than that of primary tumours (29% vs 41% respectively), and 43% of lymph node samples were negative for GIT1 compared with 25% of primary tumour samples (Table 3).

To explore this phenomenon further, we carried out a comparative analysis of GIT1 expression between the primary tumour site and matched synchronous lymph node metastasis. We found 64 cases (63%) with concordant GIT1 expression, either both positive for GIT1 expression (scores >1, n=45 (44%), or both negative for GIT1 expression (scores<1, n=19 (19%), and 38 cases (37%) with

discordant GIT1 expression between the primary tumour and the lymph node.

As intratumoural heterogeneity has been suggested to be associated with resistance to therapy and prognostic outcome in breast cancer[15], we investigated whether this spatial heterogeneity in GIT1 expression might be associated with clinical outcome (or clinicopathological characteristics) in our cohort. For this analysis we sub-classified cases into four groups: "group 0" cases were negative for GIT1 expression in both primary and lymph nodes (n=19); "group ++" cases were positive for GIT1 expression in both primary and lymph nodes (n=45); "group +/-" cases were positive for GIT1 expression in primary but not lymph nodes (i.e. loss of GIT1 expression; n=31); and "group -/+" cases were negative for GIT1 expression in primary but positive for GIT1 expression in lymph nodes (i.e. gain of GIT1 expression; n=7).

We did not detect any significant association between the aforementioned score and other clinicopathological features (Table 4). We analyzed the survival of these four groups and observed that group +/- showed a tendency towards shorter time to recurrence when compared to the rest of patients (p=0.05, hazard ratio = 2.902; Figure 2B), and that these patients had a significantly poorer overall survival than the rest of the groups (p=0.03, hazard ratio = 2.996; Figure 2C). Furthermore, the disease specific survival of patients with +/- GIT1 expression was significantly worse than other patients (p<0.0001, hazard ratio = 7.423; Figure 2D) with a median survival time of only 67 months compared to 110 months, representing a reduction of ~40%. Comparing with other well defined prognostic indicators (histological grade, presence of distant metastasis) by multivariate analysis in our cohort, the loss of GIT1 expression in lymph nodes (+/- pattern) was an independent prognostic indicator (p=0.045; (Table 5)).

In silico analysis of GIT1 expression in breast cancer

To further examine *GIT1* expression in breast cancer, we analyzed gene expression levels in several publicly available gene expression datasets. This revealed that *GIT1* expression was significantly higher in breast cancer (n=144) compared to non-tumoural tissue (n=14) (P=4.87 x 10^{-4})[8–10] (Figure 3A), and was particularly pronounced when comparing only NST cases (n=1556) with healthy breast tissue (n=144) (P=1.43 x 10^{-48}). A comparison between the two main subtypes of breast cancer (i.e. NST and ILC) in the TCGA dataset (n=525) showed differences in expression levels with healthy breast tissue (n=64) (P=5.62 x 10^{-6}) as did the dataset of Gluck *et al* (n=154 vs 4 (breast carcinoma vs healthy control) (P=0.002)) (Figure 3A)[10]. A comparison of *GIT1* expression between ER+ and ER-tumours in two independent Datasets demonstrated a significantly lower level of expression in ER+ tumours compared to ER- tumours. These comparisons were carried out on the TCGA dataset (n=601 (ER+) vs. n=179 (ER-) and the Wang dataset (n=209 (ER+) vs. n=77 (ER-)[8,11] Figure 3B). Consistent with these findings, basal tumours, which are mostly ER negative, showed higher GIT1 expression than the rest of the molecular subgroups of breast cancer in the TCGA dataset (P=6.11 x 10^{-7}; Figure 3C).

We also looked at *GIT1* expression between the primary tumour and brain metastasis in a cohort of HER2-positive (mixed ER+ and ER- cases) breast cancers (n=19)[13]. We observed that GIT1 expression was significantly decreased in brain metastases (P=0.0279; Figure 3D). Furthermore, when we interrogated data from a publicly available cohort of triple negative breast cancer patients (n=67)[12], we found that patients with GIT1 expression below the median had significantly poorer prognosis regarding event-free interval than those with GIT1 levels above the median (P=0.0411, hazard ratio = 1.625; Figure 3E).

Discussion

GIT1 is a GTPase-activating protein (GAP) that act to inhibit GTPase activity of members of the ADP-ribosylation factor (Arf) family, specifically Arf1 and Arf6, by converting bound GTP to GDP[16]. It is involved in many cellular processes including cell adhesion, migration, lamellipodia formation, cell growth and angiogenesis[17–20].

In addition, GIT1 can activate many signalling pathways involved in carcinogenesis such as ERK1/2, Rho, AARF or P21-activated kinase (PAK)[17,21]. GIT1 has been demonstrated to be over-expressed in several cancers including hepatocellular carcinoma, colon cancer, lung cancer and melanoma[17,22–25].

In the current study, we ascertained the clinical relevance of GIT1 expression by IHC in a cohort of ER positive breast cancer samples with involved synchronous lymph nodes. We observed a significant reduction in GIT1 expression in lymph node metastasis compared to matched primary breast tumours. Although Chan *et al.* reported that GIT1 is over-expressed in lymph nodes when compared to primary breast cancer, very few cases were examined by qRT-PCR (<30) and even fewer (<10) by IHC[6], the most prevalent biomarker detection technique used in clinics. Furthermore, this study did not examine the potential prognostic value of GIT1 expression or its association with distant metastasis.

Our study not only included a much larger cohort of patients (n=140) measured by IHC, but we observed the same pattern of decreased *GIT1* expression in our *in silico* analysis (see below). Moreover, a reduction in GIT1 expression in metastatic tumour cells is consistent with reports of increased Arf1 and Arf6 expression in high grade tumors compared to low grade tumors in gastric, prostate and brain, as well as in breast cancer where cell lines with high invasive activities expressed higher amounts of Arf6 protein than those in weakly invasive and non-invasive cell lines[26–28].

We observed that over a third of cases displayed a spatial intratumoural heterogeneous pattern of GIT1 expression between the primary tumour and the lymph node, with loss of GIT1 expression in lymph nodes being more common than its gain. Heterogeneity in protein expression is a well-established phenomenon in breast cancer, particularly regarding hormone receptor status, and has been associated with prognostic outcome[29]. However, these studies generally report lower levels of heterogeneity (<20%) between primary and synchrononus lymph node metastases[7,30], suggesting that GIT1 could be a more sensitive indicator of heterogeneity in this cancer, and hence a more powerful prognostic indicator. It should be noted that those cases with heterogeneous expression of GIT1 were not the same cases as

those with heterogeneous expression of hormone receptors in this cohort. Consistent with this idea, we found that loss of GIT1 in the lymph nodes (30% of patients in this study), was indicator of poor prognosis (time to recurrence and OS) by univariate analysis and an independent indicator of prognosis by multivariate analysis. It should be noted that further analysis in independent patient cohorts within a multi-centre setting is necessary to validate these findings further.

We extended these studies to other subtypes of breast cancer by looking at cohorts from publicly available databases (n= 3452) and found that GIT1 is over-expressed in breast cancer and its expression associates inversely with ER status. Furthermore, GIT1 levels were down-regulated between primary sites and distant metastases, and that was true not only in ER+ breast cancer but also other subtypes. Despite the clear evidence shown here that GIT1 is down-regulated in both lymph node and distant metastasis in breast cancer another study reported an increase in GIT1 expression between primary tumours and lymph node metastasis[6]. However, it should be borne in mind that the study of Chan *et al.* used a much smaller cohort of patients (n=26) and moreover the hormone receptor status of these patients was not provided. As our *in silico* analysis (Figure 3B) suggests that GIT1 expression varies significantly with ER status, it is plausible that the subtype analysed could influence the results. Our results support the notion that, at least in ER+ breast tumours, down-regulation of GIT1 in lymph node metastases is a sign of poor prognosis.

In summary, our study has shown that the expression of GIT1 in breast cancer could serve as a useful biomarker for the management of breast cancer patients in general and for ER+/LN+ patients in particular. The mechanistic reasons behind why the loss of GIT1 in these patients is indicative of poor prognosis remains to be determined, however it is tempting to suggest that further studies could lead to better management of these patients and ultimately improve their clinical outcome.

Data availability

Dataset 1: Clinical data of patient cohort. Table shows patients (numbered from 1 to 105) and their clinical features including histological subgroup, tumour size, number of affected lymph nodes, histological grade, vascular lymphatic infiltration, immunohistochemical initial status, treatment and patient follow up. 10.5256/f1000research.12393.d194222[31]

Dataset 2: GIT1 scoring. Table shows patients (numbered from 1 to 142) and associated primary tumour and lymph node GIT1 scoring. Categorical scores are assigned as follows according to tumour cell staining intensity; 0= negative (0%); 1=1–10%; 2= 11–50%; 3= >50%. 10.5256/f1000research.12393.d194223[32]

Dataset 3: Series mRNA expression matrix and clinical data information. GIT1 Expression Dataset consisting of 522 primary tumors, 3 metastatic tumors, and 22 tumor-adjacent normal samples. Data was median centered by genes. Platform: Affymetrix Human Genome U133A Array. Publicly available from https://tcga-data.nci.nih.gov/docs/publications/brca_2012/. 10.5256/f1000research.12393.d194224[33]

Dataset 4: Series mRNA expression matrix. Expression Dataset consisting of 2000 breast carcinoma. Platform: Affymetrix Human HT-12 V3 Array. Publicly available from http://www.cbioportal.org/study?id=brca_metabric#summary 10.5256/f1000research.12393.d194225[34]

Dataset 5: Series mRNA expression matrix. Expression Dataset consisting of one hundred fifty-four (154) invasive breast carcinoma samples and 4 normal breast samples. Platform: Agilent UNC Perou Lab Homo sapiens 1X44K Custom Array. Publicly available from Gluck Breast dataset (https://www.oncomine.org) 10.5256/f1000research.12393.d194226[35]

Dataset 6: Series mRNA expression matrix. Expression Dataset consisting of 252 lymph-node negative breast cancer samples. Platform: Affymetrix Human Genome U133A Array. Publicly available from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=gse2034 10.5256/f1000research.12393.d194227[36]

Dataset 7: Series mRNA expression matrix. Expression Dataset consisting of 67 triple negative breast cancer samples. Platform: Affymetrix Human Genome U133A Array. Publicly available from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31519 10.5256/f1000research.12393.d194228[37]

Dataset 8: Series mRNA expression matrix. Expression Dataset consisting of 19 HER2+ brain metastasis breast cancer samples and 19 HER2+ non-metastatic breast cancer samples. Platform: Affymetrix Human X3P Array. Publicly available from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43837 10.5256/f1000research.12393.d194229[38]

Consent

Written informed consent for publication of the patients' details and their images was obtained from the patients.

Competing interests

No competing interests were disclosed.

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Supplementary material

Supplementary Figure S1: Examples of GIT1 expression in whole tumour sections. Images were enhanced from the original (Supplementary Figure S3). Patient **A** demonstrates a +/- (positive primary/negative lymph node) pattern of GIT1 expression in both TMA and whole section staining. Patient **B** shows a -/+ (weak positive primary/positive lymph node) pattern of GIT1 expression. Magnification x40.

Click here to access the data.

Supplementary Figure S2: Example of GIT1 expression patterns found in ER+ breast cancer. Original representative intensity staining photos of GIT1 expression (primary tumour) of Figure 1. **A**. negative (score=0); **B**. weak (score=1); **C.** moderate (score=2); and **D.** strong (score=3). Magnification x400.

Click here to access the data.

Supplementary Figure S3: Examples of GIT1 expression in whole tumour sections. Original photos of Supplementary Figure S1. Patient **A** demonstrates a +/- (positive primary/negative lymph node) pattern of GIT1 expression in both TMA and whole section staining. Patient **B** shows a -/+ (weak positive primary/positive lymph node) pattern of GIT1 expression. Magnification x40.

Click here to access the data.

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Data Source

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Data Source

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? **Richard T. Premont**
Liver Center and Duke Institute for Brain Sciences, Duke University, Durham, NC, USA

Goicoechea et al. have examined GIT1 expression in primary breast cancers and metastatic lymph nodes a cohort of Estrogen Receptor positive (ER+) patients. While high or low GIT1 overexpression in the primary tumor does not associate with patient survival, a discordance between high GIT1 in primary tumor and low GIT1 in metastatic lymph nodes is significantly associated with reduced time to metastatic recurrence and reduced patient survival. The authors suggest that this discordance is an independent prognostic indicator of poor outcome in ER+ patients. A further analysis of published datasets shows that GIT1 mRNA level is elevated in multiple breast cancer subtypes, but this overexpression is lowest in ER+ cancers, and that low GIT1 is associated with worse outcomes in triple negative breast cancer.

The strength of the paper is in the survival curves in Figure 2, showing that the GIT1-high primary tumor/GIT1-low lymph node metastatic (+/-) subgroup is significantly worse off than patients with either always high or always low GIT1 level. This could be a very significant finding.

However, there are several major concerns:

1. The study relies on the specificity of a single GIT1 polyclonal antiserum from Bethyl. The authors should list the specific lots of antisera utilized, and describe the characterization they have performed to be assured that this antiserum is in fact detecting GIT1, and not also the related GIT2 protein or even other unrelated proteins. Is similar IHC data obtained using distinct anti-GIT1 antisera? The authors should also show and point out adjacent normal breast epithelial tissue (and lymph node tissue) for comparison. The authors should consider making all primary IHC image files available as supplemental material.

2. Regarding the metastatic lymph nodes, what evidence is there that these cells are in fact metastatic? If other markers were used, they should be mentioned. The histology of the lymph nodes in Supp Fig 1, at the magnification shown, makes it impossible to distinguish normal lymphocytes from metastatic epithelial-like cells. Again, it would be helpful to include and mark adjacent non-tumor tissue.

3. The IHC figures should include scale bars in each micrograph for size. It is particularly worrying in Figure 1A that the cell nuclei are all substantially larger than those in the other panels of that figure. The cells themselves also appear larger.

4. Table 5 appears confusing because the description is incomplete. Please clarify what was compared in each case (likely just explicitly referring to Table 4). That is, among the patient cohort,

what number and percentage of patients had distant metastasis? Define what histology grades are compared here (I vs II vs III, pooling of subgroups I and II vs III, etc)? What are +/- patients compared to, all other patients or just a subgroup? The table as shown also does not include a description of how the p=0.002 value reported in the text was obtained. Surely the Hazard ratio of 300000+ in Table 5 is a typo?

5. The discussion of the functions and cancer-associations of GIT1 is inadequate and incorrect. GIT1 is not "part of the Arf and Rho families of GTPases" as it is not a GTPase itself, but instead is a GTPase regulator (GTPase activating protein - deactivator - for Arf). GIT1 functional interactions with the Rho pathway, and with PAK, require an additional GIT1 partner, PIX, which is a Rho guanine nucleotide exchange factor (activator). The authors should carefully read two recent reviews that discuss GIT1 and cancer to distill the current understanding of GIT1 function[1,2], and then present a clearer and more accurate description of what GIT1 does and therefore why this discordance between primary and metastatic tumor might explain the differential patient survival rates they have discovered.

Minor presentation/formatting issues:

1. The authors need to be consistent in their presentation of numerical values. Values are presented in the European style with a comma separating a number from fractional values in all of the data tables ("4,843"), but in the American style using a period in the text itself ("4.843"). This is confusing.

2. The datasets are provided as Excel spreadsheets, but with all values for each patient shown within a single cell, separated by semicolons. It would make this data more immediately accessible to provide either Excel spreadsheets with the distinct values for each patient in individual cells already, or to provide this data in a less platform-dependent manner, such as CSV tables with commas between values for each patient, which can be readily imported into any statistics program.

3. In Fig 2A and Fig 3E, GIT1 is labeled as "GIT-1"

4. It is beyond the scope of this study, but an interesting mechanistic question is, since GIT1 works hand-in-hand with PIX proteins and they regulate each others' stability and expression, do PIX levels also change in GIT1 high vs low expressing tumors?

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Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Biochemical pharmacology of signal transduction pathways; discovered and characterized GIT1

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 13 Feb 2018

Ibai Goicoechea, Biodonostia, Spain

Thank you very much for the useful comments made by this reviewer which have made further enriched the revised manuscript. I hope we are able to satisfy most of your concerns regarding the manuscript. In response to the specific points:

1. *The study relies on the specificity of a single GIT1 polyclonal antiserum from Bethyl. The authors should list the specific lots of antisera utilized, and describe the characterization they have performed to be assured that this antiserum is in fact detecting GIT1, and not also the related GIT2 protein or even other unrelated proteins. Is similar IHC data obtained using distinct anti-GIT1 antisera? The authors should also show and point out adjacent normal breast epithelial tissue (and lymph node tissue) for comparison. The authors should consider making all primary IHC image files available as supplemental material.*

We agree with the reviewer that many of the conclusions drawn in this study are dependent upon the specificity of the antibodies used. However, we would also like to point out that the immunohistochemistry results were also corroborated by our *in silico* analyses. As suggested by the reviewer, the specific lot of the antisera used has been included in the revised manuscript. Regarding the possibility of cross-specificity of the Ab with GIT2, even though the antisera is polyclonal, the immunogen used (i.e. between aa 375 and aa 425) is specific to GIT1 and this sequence is not present in GIT2. Furthermore, this antibody is the same that was used in the study of Chan et al (Oncogene 2014). That said we cannot rule out non-specific staining due to cross-reactivity against other proteins. This caveat has been made clear in the revised manuscript. Unfortunately, due to routine surgical protocols adjacent normal breast epithelial tissue was not available for these cases.

1. *Regarding the metastatic lymph nodes, what evidence is there that these cells are in fact metastatic? If other markers were used, they should be mentioned. The histology of the lymph nodes in Supp Fig 1, at the magnification shown, makes it impossible to distinguish*

normal lymphocytes from metastatic epithelial-like cells. Again, it would be helpful to include and mark adjacent non-tumor tissue.

The identification of metastatic lymph nodes was carried out on the basis of H&E stains, by an expert breast cancer pathologist with more than 25 years' experience. We agree it would be very useful to include adjacent non-tumor tissue in this study however this material was not available as immunohistochemical staining was carried out on TMAs rather than whole sections and cores were selected on the basis of high-tumour content so did not contain a significant non-tumoral component.

3. The IHC figures should include scale bars in each micrograph for size. It is particularly worrying in Figure 1A that the cell nuclei are all substantially larger than those in the other panels of that figure. The cells themselves also appear larger.

The figures all represent magnified fields at 400x magnification and scale bars have been added to the figure as suggested.

1. *Table 5 appears confusing because the description is incomplete. Please clarify what was compared in each case (likely just explicitly referring to Table 4). That is, among the patient cohort, what number and percentage of patients had distant metastasis? Define what histology grades are compared here (I vs II vs III, pooling of subgroups I and II vs III, etc)? What are +/- patients compared to, all other patients or just a subgroup? The table as shown also does not include a description of how the p=0.002 value reported in the text was obtained. Surely the Hazard ratio of 300000+ in Table 5 is a typo?*

We agree that the description of Table 5 could be confusing and have therefore changed the description accordingly.

1. *The discussion of the functions and cancer-associations of GIT1 is inadequate and incorrect. GIT1 is not "part of the Arf and Rho families of GTPases" as it is not a GTPase itself, but instead is a GTPase regulator (GTPase activating protein - deactivator - for Arf). GIT1 functional interactions with the Rho pathway, and with PAK, require an additional GIT1 partner, PIX, which is a Rho guanine nucleotide exchange factor (activator). The authors should carefully read two recent reviews that discuss GIT1 and cancer to distill the current understanding of GIT1 function[1,2], and then present a clearer and more accurate description of what GIT1 does and therefore why this discordance between primary and metastatic tumor might explain the differential patient survival rates they have discovered.*

We thank the reviewer for pointing out these references and the information contained within. The discussion has been re-written to reflect the comments of the reviewer.

Minor presentation/formatting issues:

1. The authors need to be consistent in their presentation of numerical values. Values are presented in the European style with a comma separating a number from fractional values in all of the data tables ("4,843"), but in the American style using a period in the text itself ("4.843"). This is confusing.

This has been changed in the revised manuscript

1. The datasets are provided as Excel spreadsheets, but with all values for each patient shown within a single cell, separated by semicolons. It would make this data more immediately accessible to provide either Excel spreadsheets with the distinct values for each patient in

individual cells already, or to provide this data in a less platform-dependent manner, such as CSV tables with commas between values for each patient, which can be readily imported into any statistics program.

This has been changed in the revised manuscript

1. In Fig 2A and Fig 3E, GIT1 is labeled as “GIT-1”
2. It is beyond the scope of this study, but an interesting mechanistic question is, since GIT1 works hand-in-hand with PIX proteins and they regulate each others’ stability and expression, do PIX levels also change in GIT1 high vs low expressing tumors?

Competing Interests: no competing interests

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? **Mona M. Mohamed**
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Abstract

The authors mentioned that "GIT1 expression in tumour cells was scored and statistical correlation analyses were carried out". Authors should point with arrow to all tumour cells showing stain of GIT1 in tissue and differentiate between expression of GIT1 by cancer cells and stromal cells.

"We observed that loss fo GIT1 expression in the metastasis was associated with a shorter time to recurrence, poorer overall survival, and a shorter median survival time."

Authors should explain here that loss of "GIT1 expression" by which cells? Tumor metastatic cells in the lymph node or by cell populations of the lymph nodes (example: immune cells)?

In material and methods scoring of GIT1 is only in tumor cells so which cells are scored in lymph nodes. Please note that the cell populations of lymph nodes (LNs) is different from that of the tumor microenvironment and authors depends on scoring of GIT1 expression by the tumor cells (less in LNs) so this should be clarified by authors, since the IHC microscopic images provided is confusing and may show non-specific stain.

Methods

"All primary site tumours were ER+ and 91% of them PR+."
The authors should include ER- (ER negative) patients as a control to the studied group to provide their hypothesis that ’the expression of GIT1 in breast cancer could serve as a useful biomarker for the management of breast cancer patients in general and for ER+/LN+ patients in particular."

Although the paper depends mainly on IHC the auhtors did not mention appropriate chromogen/substrate used to develop the color visualized using the microscope. This section should be written in details.

I cannot read the Dataset files provided by the author, the columns and rows overlap.

Statistical analysis
"Chi-square statistical test was used to determine association between GIT1 expression and lymph node metastasis (Table 3)"

What type of correlation test used here?

Do the authors correlate between GIT1 expression in primary tumor and the "number of metastatic lymph node" this section should be clarified?

Results
The Supplementary Figures S1, S2 and S3 representing microscopic images for GIT1 show non-specific stains. The authors should present better microscopic images with high magnification showing the pattern of GIT1 by carcinoma cells and stromal cells.

"Spatial intratumoural heterogeneity of GIT1 expression in ER+ breast cancer: When we compared GIT1 expression in primary tumours with that of their counterpart lymph node metastases surprisingly we observed a significant decrease of GIT1 expression (p=0.0054, Table 3)."

The authors did not take in consideration that cellular population of LN is different from that of carcinoma cells. A better IHC images should be presented showing which cell population express GIT1 in each of carcinoma tissues and lymph nodes.

"A comparison of GIT1 expression between ER+ and ER- tumours in two independent Datasets demonstrated a significantly lower level of expression in ER+ tumours compared to ER- tumours (8,11)."

The references (8,11); assess GIT1 at mRNA level not at the protein level, in the present study authors should include ER- patients and their associated lymph nodes as control group to ER+.

Discussion
Should be re-written presenting the mechanisms of GIT1 in carcinogenesis and scientific explanation for the authors findings "loss of GIT1 expression in the metastasis was associated with a shorter time to recurrence, poorer overall survival, and a shorter median survival time".

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
No

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Cancer biology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Jan 2018

Ibai Goicoechea, Biodonostia, Spain

We thank the reviewer for their insightful comments and have made changes in the revised manuscript to address these concerns. Specifically:

Abstract
P1: The authors mentioned that "GIT1 expression in tumour cells was scored and statistical correlation analyses were carried out". Authors should point with arrow to all tumour cells showing stain of GIT1 in tissue and differentiate between expression of GIT1 by cancer cells and stromal cells.

Only tumor cells were scored in this analysis not stromal or other microenvironment cell types

"We observed that loss fo GIT1 expression in the metastasis was associated with a shorter time to recurrence, poorer overall survival, and a shorter median survival time."
Authors should explain here that loss of "GIT1 expression" by which cells? Tumor metastatic cells in the lymph node or by cell populations of the lymph nodes (example: immune cells)?

Tumor cells (text changed in revised manuscript)

In material and methods scoring of GIT1 is only in tumor cells so which cells are scored in lymph nodes. Please note that the cell populations of lymph nodes (LNs) is different from that of the tumor microenvironment and authors depends on scoring of GIT1 expression by the tumor cells (less in LNs) so this should be clarified by authors, since the IHC microscopic images provided is confusing and may show non-specific stain.

Only tumor cells were scored in the involved lymph nodes, non-involved lymph nodes were used as negative controls in this instance.

Methods
"All primary site tumours were ER+ and 91% of them PR+."
The authors should include ER- (ER negative) patients as a control to the studied group to provide their hypothesis that 'the expression of GIT1 in breast cancer could serve as a useful biomarker for the management of breast cancer patients in general and for ER+/LN+ patients in particular."

We agree that this would have been a good comparison to carry out. Unfortunately there

were insufficient suitable ER- cases available for testing. We also decided to focus the study on ER+ cases as representing the majority of breast cancer cases

Although the paper depends mainly on IHC the auhtors did not mention appropriate chromogen/substrate used to develop the color visualized using the microscope. This section should be written in details.

The chromagen used was DAB. This information has been included in the revised manuscript

I cannot read the Dataset files provided by the author, the columns and rows overlap.

I have informed the editor of this problem

Statistical analysis

"Chi-square statistical test was used to determine association between GIT1 expression and lymph node metastasis (Table 3)"

What type of correlation test used here?

This was Chi-square analysis

Do the authors correlate between GIT1 expression in primary tumor and the "number of metastatic lymph node" this section should be clarified?

This is an interesting point and we have added this analysis to the revised manuscript. There was no correlation.

Results

The Supplementary Figures S1, S2 and S3 representing microscopic images for GIT1 show non-specific stains. The authors should present better microscopic images with high magnification showing the pattern of GIT1 by carcinoma cells and stromal cells.

Whilst we agree with the reviewer that some GIT1 expression was non-specific this was very different staining from that seen in tumor tissue. This can be seen clearly from the figures S1 and a high magnification image is already given in Figure 1 whereby some light stromal staining is seen in the panel A compared to string tumour staining in panel D.

"Spatial intratumoural heterogeneity of GIT1 expression in ER+ breast cancer: When we compared GIT1 expression in primary tumours with that of their counterpart lymph node metastases surprisingly we observed a significant decrease of GIT1 expression (p=0.0054, Table 3)."

The authors did not take in consideration that cellular population of LN is different from that of carcinoma cells. A better IHC images should be presented showing which cell population express GIT1 in each of carcinoma tissues and lymph nodes.

We disagree with the reviewer that we did not take into consideration the difference in non-tumoral staining between lymph node and primary material. The scoring was carried

out by a breast pathologist with more than 25 years experience in the field. These differences are clearly shown in Supplementary Figure S1 which shows representative images of negative and positive lymph node staining, as well as primary tumor material.

"A comparison of GIT1 expression between ER+ and ER- tumours in two independent Datasets demonstrated a significantly lower level of expression in ER+ tumours compared to ER- tumours (8,11)."

The references (8,11); assess GIT1 at mRNA level not at the protein level, in the present study authors should include ER- patients and their associated lymph nodes as control group to ER+.

As stated above we would have liked to included ER- cases as well. However, that was not possible

Discussion

Should be re-written presenting the mechanisms of GIT1 in carcinogenesis and scientific explanation for the authors findings "loss of GIT1 expression in the metastasis was associated with a shorter time to recurrence, poorer overall survival, and a shorter median survival time".

The discussion has been rewritten to add possible explanations for this statement

Competing Interests: no competing interests

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