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Original Articles

GATA3 mRNA expression, but not mutation, associates with longer progression-free survival in ER-positive breast cancer patients treated with first-line tamoxifen for recurrent disease

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ABSTRACT

In breast cancer, *GATA3* mutations have been associated with a favorable prognosis and the response to neoadjuvant aromatase inhibitor treatment. Therefore, we investigated whether *GATA3* mutations predict the outcome of tamoxifen treatment in the advanced setting. In a retrospective study consisting of 235 hormone-naive patients with ER-positive breast cancer who received tamoxifen as first-line treatment for recurrent disease, *GATA3* mutations (in 14.0% of patients) did not significantly associate with either the overall response rate (ORR) or with the length of progression-free survival (PFS) after the start of tamoxifen therapy. Interestingly, among 148 patients for whom both mutation and mRNA expression data were available, *GATA3* mutations associated with an increased expression of *GATA3*. However, only 23.7% of *GATA3* high tumors had a mutation. Evaluation of the clinical significance of *GATA3* mRNA revealed that it was associated with prolonged PFS, but not with the ORR, also in multivariate analysis. Thus, *GATA3* mRNA expression, but not *GATA3* mutation, is an independent predictor of prolonged PFS in ER-positive breast cancer patients who received first-line tamoxifen for recurrent disease. Besides GATA3 mutation, other mechanisms must exist that underlie increased *GATA3* levels.

Introduction

Breast cancer is one of the most frequently diagnosed cancers in Western women. About 70% of all diagnosed breast cancers are estrogen receptor α (ER) positive. ER-positive breast cancers are well-differentiated and have a better outcome compared to other subtypes [1,2]. In this respect, tamoxifen is a frequently used and effective drug for patients diagnosed with ER-positive disease. However, half of ER-positive patients who receive tamoxifen as first-line therapy for recurrent disease do not respond to the treatment due to intrinsic resistance, while the other half initially responding patients become resistant during treatment [3]. To better understand the mechanism involved in this intrinsic and acquired resistance and to be able to predict which patients are likely to respond to tamoxifen, the identification of novel markers predicting the efficacy of tamoxifen treatment is highly needed.

GATA3 belongs to a family of zinc-finger transcription factors and is involved in embryogenesis and the differentiation of a variety of human tissues, including kidney, skin, breast and the central nervous system [4–8]. Both in the normal mammary gland and breast cancer tissue, GATA3 and ER expression are highly correlated [6,9]. In fact, GATA3 is expressed in the normal luminal epithelial cells where it maintains luminal cell differentiation [7], whereas in breast cancer *GATA3* is highly expressed in the luminal subtype, regulating differentiation and suppressing dissemination [7,10,11]. Furthermore, GATA3 is an integral component of the ER pathway as it regulates the pioneer factor FOXA1 and mediates ER binding by shaping enhancer accessibility [7,12]. Consequently, a large overlap exists between co-expressed genes for ER and GATA3, including many well-known ER pathway genes [13]. Since the expression of ER has important implications for both prognosis and treatment of breast cancers, several studies have assessed the association of GATA3 with clinical outcome. High GATA3 protein expression was shown to be associated with a

Abbreviations: CI, confidence interval; ER, estrogen receptor α; ERBB2, epidermal growth factor receptor 2; GATA3, GATA-binding protein 3; HR, hazard ratio; MAF, minor allele frequency; OR, odds ratio; ORR, overall response rate; PFS, progression-free survival; PR, progesterone receptor; RT-PCR, reverse transcriptase PCR; RT-qPCR, reverse transcriptase quantitative PCR.

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lower grade, smaller tumor size and increased ER and PR expression [14–18]. In line with these findings, some, but not all, studies have shown that both *GATA3* mRNA and GATA3 protein expression are independent prognostic markers, where high levels of GATA3 associate with a longer disease-free and overall survival in breast cancer patients [14,15,18–21]. Furthermore, in a small study of Parikh et al. high levels of GATA3 protein were predictive of hormone responsiveness in ER-positive breast cancer patients [22]. In the neoadjuvant setting both *GATA3* mRNA and GATA3 protein expression were shown to be predictive of a favorable response to chemotherapy [17,23].

The human *GATA3* gene is a highly conserved gene located at 10p14-15 and consists of six exons which encode a protein of 444 residues [24]. Germline mutations of *GATA3* cause a rare and complex disease of hypoparathyroidism, sensorineural deafness and renal insufficiency (HDR syndrome) [25]. In breast cancer, *GATA3* is one of the most frequently mutated genes [26–29] and sporadic heterozygous *GATA3* mutations have been identified in approximately 5–20% of ER-positive breast cancers [30]. These mutations mostly cluster in the vicinity of the second zinc finger of GATA3 [31] and are virtually absent among ER-negative breast cancers. Interestingly, *GATA3* mutations were correlated with improved disease-free and overall survival in breast cancer patients overall, but also in ER-positive breast cancer patients who received adjuvant endocrine therapy [32]. Furthermore, mutations in *GATA3* were also shown to be correlated with response to neoadjuvant aromatase inhibition treatment [33]. This suggests that *GATA3* mutation may be a determinant of the response to hormonal treatment.

To investigate this hypothesis, we analyzed exons 5 and 6 of the *GATA3* gene for mutations in 235 ER-positive primary breast cancers and evaluated the association of the identified mutations with the ORR and PFS of first-line tamoxifen therapy given for recurrent disease, as well as with *GATA3* mRNA expression levels.

Materials and methods

Study population

This retrospective study included 235 female breast cancer patients (Fig. 1) and was approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam, the Netherlands (MEC 02.953). In this study we adhered to the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl) and results are reported in accordance with the REMARK criteria on clinical reporting [34]. All patients were diagnosed between 1979 and 1996 with measurable breast cancer disease, underwent primary surgery and were treated with tamoxifen as first-line treatment that was given for recurrent disease. Primary tumors were ER-positive and a minimum of 100 mg of fresh frozen tissue was required for downstream DNA and RNA extraction [35]. ER and/or PR positivity was defined by ≥10 fmol/mg cytosolic protein [36,37] and *ERBB2* overexpression was defined by a reverse transcriptase quantitative PCR (RT-qPCR) expression level ≥18 [38]. The patients did not receive neo-adjuvant therapy or adjuvant hormonal treatment, did not experience previous other cancers and did not show subjective or objective toxicity [35]. There were 296 patients that fulfilled these criteria and from whom detailed clinical follow up and primary tumor DNA was available. However, 60 patients were excluded as the percentage of tumor cell nuclei was below 50%, precluding reliable mutation analysis. Furthermore, mutation analysis failed in 1 patient, totaling to n = 235 included in the present study. From these, 84 patients underwent breast-conserving lumpectomy and 151 underwent modified mastectomy. In addition, 17 patients received adjuvant anthracycline-containing chemotherapy and 14 received adjuvant chemotherapy without anthracyclines. There were 209 M0 patients and 26 M1 patients. The median age at the time of the primary surgery was 57 years, while the median age at the start of first-line treatment was 61 years. Criteria for follow up and response to tamoxifen therapy were defined by standard International Union Against Cancer criteria of tumor response [39]. Complete and partial remission (together objective response) was observed in 4 and 34 patients, respectively, whereas 52 patients had progressive disease. From the patients with stable disease, 132 had no change for longer than 6 months, whereas 13 patients had no change for ≤6 months. According to the advice of the European Organization for Research and Treatment of Cancer [40], we defined overall response as complete and partial remission including stable disease >6 months. As a result, 170 patients were classified as responders to tamoxifen and 65 patients showed no response to tamoxifen. The median follow up of patients after the start of tamoxifen therapy was 49 months (range: 4–208 months). At the end of the follow up, 224 patients had developed tumor progression and 196 patients had died.

From 148 of the 235 patients we had total RNA of sufficient quality from the primary tumor available (*i.e.* at an input of 10 ng total RNA amplifiable for 3 reference genes within 25 cycles) in order to perform *GATA3* mRNA expression analysis by RT-qPCR. The clinicopathological variables of the patients are shown in Table 1.

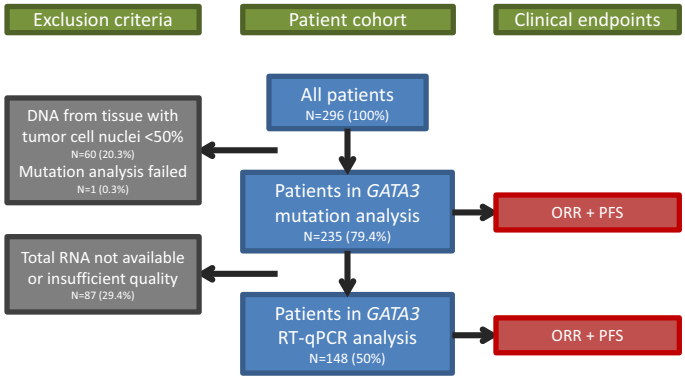

Fig. 1. Study design and patient subsets analyzed for *GATA3* mutation status and *GATA3* mRNA expression. The "All patients (N = 296)" box represents all hormone-naive patients diagnosed with recurrent breast cancer between 1979 and 1996 and were treated with first-line tamoxifen (details provided in the Materials and methods section). For the *GATA3* mutation analysis 60 patients were excluded whose tumor cell nuclei percentage was below 50% and mutation analysis failed in one patient. From these 235 patients, total RNA of sufficient quality was available for 148 patients for *GATA3* RT-qPCR analysis. Clinical endpoints were the overall response rate (ORR) and progression-free survival (PFS).

Mutation analysis

Genomic DNA previously extracted from the fresh frozen primary breast tumor of 235 patients [41] and quantified by Picogreen was used at an input of 20 ng to amplify *GATA3* exon 5 and 6 sequences. Subsequently, PCR amplicons were subjected to Sanger sequencing analysis on an ABI3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA). All mutations were confirmed by Sanger sequencing using an independently amplified template. For the splice acceptor site mutations we performed an exonic reverse transcriptase PCR (RT-PCR) on a RNA template instead of PCR on a DNA template. We reported *GATA3* mutations and predicted protein changes according the HGVS recommendations for the description of sequence variants [42]. PCR and sequencing primer sequences are available in Table S1A and B.

Expression analysis

Total RNA was extracted and cDNA was synthesized previously from the fresh frozen primary breast tumor of 148 patients as described before [35]. qPCR for *GATA3* was performed in a Mx3000P™ Real-Time PCR System (Agilent, Amsterdam, the Netherlands) using SensiFast Probe Lo-Rox master mix (GC Biotech, Alphen aan den Rijn, the Netherlands) and a Taqman Gene expression Assay kit from Applied Biosystems (Hs00231122_m1; spanning exons 2 to 3; Nieuwerkerk aan den IJssel, the Netherlands) with 40 rounds of amplification as recommended by the manufacturer. In addition to a negative control (*i.e.* genomic DNA), we also included a standard curve of a serially diluted cDNA sample consisting of pooled breast cancer cDNA samples in each PCR plate. The latter was done to ensure that the PCR efficiency between plates was comparable and to normalize the data obtained from different plates and experiments. *GATA3* mRNA expression levels for the samples were determined relative to the average Cq value of our reference gene set consisting of hydroxymethylbilane synthase (*HMBS*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and TATA-box binding protein (*TBP*), and quantified as follows: *GATA3* expression = $2^{\text{Cq reference gene set} - \text{Cq } GATA3 \text{ gene}}$ [35]. PCR primer sequences for the reference genes are available in Table S1C.

Statistical analyses

A χ^2 or a Fisher's exact test (when the expected frequency ≤5 in any of the groups) was used to evaluate the relation between *GATA3* mutation status and the clinicopathological variables. The relation between *GATA3* mRNA expression levels and the clinicopathological variables was evaluated using either the two-sample Wilcoxon rank-sum test (for 2 categories) or the Kruskal–Wallis equality-of-populations rank test (for 3 categories). The association with tamoxifen response was analyzed with a logistic regression model to calculate odds ratios (ORs) and their 95% confidence intervals (95% CIs). PFS analysis was performed by the Kaplan–Meier method for visualization purposes and differences between survival curves were calculated by the Peto & Peto modification of the Gehan–Wilcoxon test (which puts more weight on the earlier events) for *GATA3* mutation status and the log-rank test for *GATA3* gene

Table 1
Association of *GATA3* mutation status and *GATA3* gene expression levels with clinicopathological variables in 235 ER-positive primary breast cancers.

Variable	*GATA3* mutation status			*GATA3* expression level		
	Number of wild-type patients	Number of mutant patients	*P*-value	Number of patients	Median *GATA3* expression[a]	*P*-value
Total number	202	33		148	1.224	
Menopausal status[b]			0.22			0.25
Premenopausal	44	11		33	0.891	
Postmenopausal	157	22		115	1.238	
Tumor grade			0.68			0.24
Good/Moderate	26	5		17	1.723	
Poor	116	15		86	1.010	
Unknown	60	13		45	1.242	
Tumor size			0.057			0.41
pT1	54	4		39	1.106	
pT2 + unknown	120	27		93	1.276	
pT3 + pT4	28	2		16	2.053	
Nodal status			0.36			0.37
N0	83	18		71	1.287	
N1–3	43	7		30	1.172	
N > 3	63	7		37	0.767	
Unknown	13	1		10	0.778	
Dominant site of relapse			0.25			0.019
Soft	24	1		13	0.276	
Bone	107	17		83	1.463	
Visceral	71	15		52	0.872	
Disease-free interval (m)			0.53			0.42
≤12	53	8		39	1.225	
13–36	78	16		64	1.144	
>36	71	9		45	1.340	
PR protein status			0.28			0.0015
Positive	155	22		116	1.082	
Negative	46	11		32	1.646	
Unknown	1	0				
ERBB2 mRNA status			0.54			0.084
Positive	20	2		18	0.807	
Negative	149	29		130	1.259	
Unknown	33	2				

[a] Log2-transformed *GATA3* gene expression levels.
[b] At the start time of first-line tamoxifen treatment; m, months.

expression. Univariate and multivariate Cox proportional hazards regression models were applied to calculate the hazards ratios (HRs) and 95% CIs in the analysis for the PFS. Log2-transformed expression values for *GATA3* mRNA, ER and PR protein and *ERBB2* mRNA were used in logistic and Cox regression analyses. All *P*-values were two-sided and *P*-values smaller than 0.05 were considered to be statistically significant. Analyses were performed using R, version 3.2.3.

Results

Since at the start of this study all of the *GATA3* mutations reported so far clustered in exons 5 and 6 of the *GATA3* gene, which encode the highly conserved second zinc finger required for DNA binding, we limited sequence analysis to these two exons. In total, we identified at least one *GATA3* sequence variant in 54 out of the 235 primary tumors of patients with ER-positive recurrent breast cancer. A silent mutation in exon 5 (rs11567941; c.1257G>A; p.T122T; minor allele frequency (MAF) = 0.02) was identified in 24 tumors; however, we did not consider this mutation to be pathogenic. Furthermore, we identified a frameshift insertion in 22 tumors, a frameshift deletion in five tumors and a splice site deletion in six tumors (Table 2). These mutations predicted prematurely truncated proteins in 14 tumors and proteins with a longer C-terminal tail in 19 tumors. In total, we identified 33 *GATA3* mutations that we considered to be pathogenic in 33 (14.0%) out of 235 ER-positive primary breast tumors.

Next, we evaluated the association between *GATA3* mutation status and the clinicopathological variables (Table 1), the ORR (Table S2) and the length of PFS after start of tamoxifen treatment (Table S2 and Fig. 2A). We found no relation between *GATA3* mutation and any of the clinicopathological variables (Table 1).

Table 2
Identified *GATA3* mutations among 235 ER-positive primary breast cancers.

Location	Nucleotide change	Predicted protein change	Number of patients
Exon 5	c.925-3_925-2delCA	p.S309Pfs*45	6
Exon 5	c.961_962delTG	p.C321Sfs*31	1
Exon 5	c.983_984insC	p.W329Lfs*25	1
Exon 5	c.1002_1003insG	p.G335Gfs*18	1
Exon 5	c.1003delG	p.G335Gfs*20	1
Exon 5	c.1007_1008insC	p.V341Vfs*15	1
Exon 5	c.1021_1022insC	p.A341Afs*11	1
Exon 5	c.1033_1034insAC	p.Y345Yfs*11	1
Exon 5	c.1035_1036insT	p.Y346Lfs*7	1
Exon 6	c.1195_1196delAG	p.R399Tfs*108	1
Exon 6	c.1202_1203insG	p.S402Vfs*106	1
Exon 6	c.1202_1203insGTCC	p.S403Vfs*106	1
Exon 6	c.1206_1207insT	p.S403Ffs*105	2
Exon 6	c.1207_1208insC	p.L404Pfs*103	1
Exon 6	c.1222_1223insC	p.P409Afs*99	2
Exon 6	c.1223_1224insT	p.P409Sfs*100	1
Exon 6	c.1223_1224insG	p.P409Afs*99	1
Exon 6	c.1257_1258insC	p.T421Hfs*87	1
Exon 6	c.1263_1282del20	p.M423Vfs*78	1
Exon 6	c.1271_1272insC	p.P425Afs*82	2
Exon 6	c.1277_1278insA	p.S427Ifs*81	2
Exon 6	c.1304_1305insC	p.S437Lfs*71	2
Exon 6	c.1305delC	p. S437Pfs*39	1

Nomenclature for the identified nucleotide changes and predicted protein changes is according the HGVS recommendations for the description of sequence variants [42].
* Stop codon.

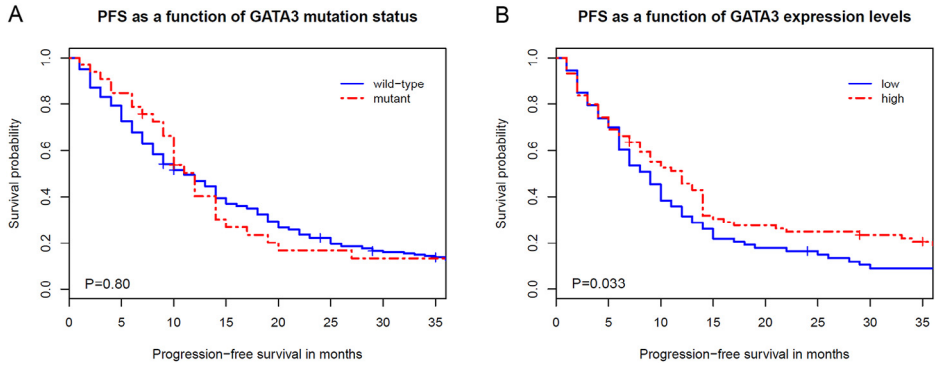

Fig. 2. Kaplan–Meier analysis of progression-free survival (A) according to *GATA3* mutation status for 235 ER-positive breast cancer patients who received first-line tamoxifen therapy for recurrent disease. The difference between the survival curves was calculated using the Peto & Peto modification of the Gehan–Wilcoxon test. (B) Dichotomized at median *GATA3* expression level for 148 ER-positive breast cancer patients who received first-line tamoxifen therapy for recurrent disease. The difference between the survival curves was calculated using the log-rank test.

Furthermore, *GATA3* mutations did not significantly associate with the ORR for tamoxifen therapy in univariate logistic regression analysis (70.8% versus 81.8%; OR = 1.86, 95% CI = 0.73–4.73, $P = 0.19$; Table S2) or with the length of PFS in Kaplan–Meier ($P = 0.80$; Fig. 2A) and Cox regression analysis (HR = 0.95, 95% CI = 0.65–1.40, $P = 0.81$; Table S2). Also subsetting by the type of mutation (*i.e.* mutations predicted to truncate versus elongate the protein) did not yield any significant differences. Although the number of patients was small, survival curves appeared very similar. However, logistic regression analysis did show that the traditional predictive factor disease-free interval was associated with the efficacy of tamoxifen therapy (Table S2). Similarly, the traditional predictive factors dominant site of relapse, disease-free interval and the level of PR protein expression were found to be associated with PFS (Table S2). These results implied that *GATA3* mutation status is not a significant predictor for the outcome of tamoxifen therapy in patients with recurrent disease.

Out of the 235 tumors for which we performed *GATA3* mutation analysis, we were able to perform *GATA3* mRNA expression analysis by RT-qPCR for 148 tumors. In 25 out of these 148 tumors we identified a *GATA3* mutation and 123 tumors were wild-type. Interestingly, *GATA3* expression levels were higher among mutant *GATA3* tumors (*i.e.* irrespective of the predicted effect of the mutation) than wild-type *GATA3* tumors ($P = 0.0019$). Eighteen tumors (72.0%) with *GATA3* mutations had high *GATA3* expression levels (*i.e.* above the median) while only seven tumors (28.0%) with *GATA3* mutations had low *GATA3* expression levels (*i.e.* below the median). However, out of the 76 *GATA3* high expressing tumors, only 18 (23.7%) had a mutation in the *GATA3* gene. Because the high levels of *GATA3* mRNA were only partially explained by a mutation in *GATA3* itself, we hypothesized that *GATA3* expression instead of mutation might be associated with the outcome of tamoxifen treatment.

To evaluate this, we made use of the *GATA3* mRNA expression data for all 148 primary breast tumors from recurrent breast cancer patients that we had generated by RT-qPCR. We found that *GATA3* expression was associated with dominant site of relapse and PR protein status, but not with menopausal status, tumor grade, tumor size, nodal status, disease-free interval or *ERBB2* mRNA status (Table 1). In univariate logistic regression analysis, we found no association of *GATA3* expression level with the ORR for tamoxifen (64.9% versus 66.2%; OR = 1.12, 95% CI = 0.90–1.41, $P = 0.31$; Table 3). Additionally, menopausal status, dominant site of relapse and ER protein, PR protein and *ERBB2* mRNA expression levels were also not associated with the ORR for tamoxifen, in contrast to disease-free interval (Table 3). *GATA3* expression was, however, associated with the length of PFS, as it was prolonged for patients with tumors with high *GATA3* mRNA levels compared to those with low levels ($P = 0.033$; Fig. 2B). Concordantly, in univariate Cox regression analysis, high *GATA3* expression levels were significantly associated with

Table 3
Univariate logistic regression analysis of the overall response rate in 148 ER-positive breast cancer patients treated with first-line tamoxifen for recurrent disease.

Variable	Univariate analysis	
	OR (95% CI)	*P*-value
Base model:		
Menopausal status[a]		
Premenopausal	1	
Postmenopausal	1.83 (0.83–4.03)	0.13
Dominant site of relapse		
Soft	1	
Bone	0.43 (0.11–1.69)	0.23
Visceral	0.81 (0.20–3.40)	0.78
Disease-free interval (m)		
≤12	1	
13–36	4.70 (1.98–11.11)	0.00043
>36	3.54 (1.43–8.76)	0.0063
ER protein expression	1.12 (0.95–1.33)	0.17
PR protein expression	1.06 (0.95–1.18)	0.30
ERBB2 mRNA expression	1.07 (0.95–1.22)	0.26
Additions to the base model:		
GATA3 mRNA expression	1.12 (0.90–1.41)	0.31

[a] At the start time of first-line tamoxifen treatment; m, months.

Table 4
Univariate and multivariate Cox regression analyses of progression-free survival in 148 ER-positive breast cancer patients treated with first-line tamoxifen for recurrent disease.

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Base model:				
Menopausal status[a]				
Premenopausal	1		1	
Postmenopausal	0.73 (0.49–1.09)	0.12	0.84 (0.54–1.29)	0.42
Dominant site of relapse				
Soft	1		1	
Bone	1.88 (0.99–3.57)	0.054	2.36 (1.21–4.63)	0.012
Visceral	1.47 (0.76–2.86)	0.26	1.74 (0.86–3.53)	0.13
Disease-free interval (m)				
≤12	1		1	
13–36	0.66 (0.43–0.99)	0.046	0.61 (0.40–0.93)	0.021
>36	0.56 (0.36–0.88)	0.012	0.59 (0.37–0.94)	0.026
ER protein expression	0.95 (0.87–1.03)	0.22	0.97 (0.88–1.06)	0.49
PR protein expression	0.95 (0.90–1.00)	0.051	0.95 (0.89–1.01)	0.077
ERBB2 mRNA expression	0.98 (0.91–1.05)	0.62	0.98 (0.91–1.06)	0.62
Additions to the base model:				
GATA3 mRNA expression	0.87 (0.78–0.98)	0.017	0.85 (0.75–0.96)	0.0079

[a] At the start time of first-line tamoxifen treatment; m, months.

a prolonged PFS (HR = 0.87, 95% CI = 0.78–0.98, $P = 0.017$; Table 4). Besides *GATA3* expression levels, also disease-free interval, but not menopausal status, dominant site of relapse and ER protein, PR protein and *ERBB2* mRNA expression levels, were associated with the length of PFS (Table 4). In multivariate analysis, by including *GATA3* expression in a model with all the traditional predictive factors, *GATA3* expression levels were significantly associated with a prolonged PFS (HR = 0.85, 95% CI = 0.75–0.96, $P = 0.0079$; Table 4). These results imply that *GATA3* mRNA expression, rather than genetic aberration of the gene alone, is an independent predictor for the length of PFS in hormone-naive ER-positive breast cancer patients treated with first-line tamoxifen for recurrent disease.

Discussion

GATA3 is one of the most frequently mutated genes in breast cancer [26–29] and mutations in *GATA3* are associated with improved survival [32]. Because *GATA3* mutations are also associated with both a favorable outcome among ER-positive patients who received adjuvant endocrine treatment as well as response to neoadjuvant aromatase inhibitors [32,33], we here evaluated whether *GATA3* mutations measured in the primary tumor (*i.e.* all ER positive) can determine the outcome of patients treated with first-line tamoxifen for recurrent disease. However, *GATA3* mutations were not significantly associated with either the ORR or with PFS in 235 ER-positive breast cancer patients who received tamoxifen as a first-line therapy for recurrent disease (Table S2 and Fig. 2A). Even though *GATA3* mutations were associated with increased levels of *GATA3* expression, only *GATA3* expression was found to be an independent predictor for prolonged PFS (Table 4). Our results suggest that not *GATA3* mutation, but rather *GATA3* expression predicts the length of PFS. This result will need to be validated in an independent patient population.

Jiang et al. have previously shown that *GATA3* mutations were associated with improved survival in both the TCGA cohort as well as the Chinese FUSCC cohort [32]. In the TCGA cohort, however, this prognostic effect was limited to ER-positive breast cancer cases (overall survival $P = 0.041$) in contrast to all cases in the FUSCC cohort (overall survival $P = 0.033$). Furthermore, in the FUSCC cohort, *GATA3* mutations were also associated with longer disease-free survival in ER-positive patients who received adjuvant endocrine treatment ($P = 0.046$), which may suggest a role for *GATA3* mutation in the efficacy of endocrine therapy. However, our results do not show that *GATA3* mutations are associated with the outcome of tamoxifen treatment in 235 ER-positive patients who were treated with first-line tamoxifen for recurrent disease (Table S2 and Fig. 2A). This suggests that the improved disease-free survival of patients with *GATA3* mutated tumors in the FUSCC cohort can be attributed to a pure prognostic association of *GATA3* mutation rather than its role as a predictive factor for tamoxifen efficacy. Noteworthy, however, is that *GATA3* mutations in the neoadjuvant setting were a predictive marker of favorable outcome of aromatase inhibitor treatment [33]. In that study, 77 ER-positive breast cancer samples were sequenced and *GATA3* mutations were more frequently present in aromatase inhibitor sensitive tumors ($P = 0.01$). The apparent discrepancy between our study and the study of Ellis et al. might be attributable to a difference in the mechanism of action between aromatase inhibitors and tamoxifen or, probably more likely, due to the difference in primary versus recurrent disease receiving endocrine treatment and the used endpoints.

At the gene expression level, high *GATA3* has consistently and independently of other clinicopathological predictors been linked to a better outcome [14,19], but at the protein level the prognostic effect of GATA3 remains controversial [15,18,20,21]. Higher sensitivity and/or accuracy of gene expression compared with protein expression measurement methods could very well explain poor consistency at the protein level. Interestingly, in a small study including only 28 patients and examining the expression of GATA3 by immunohistochemistry, Parikh et al. found that GATA3 expression predicted hormone responsiveness in breast cancer as six of 14 (43%) cancers were GATA3 negative in the hormone-unresponsive group and 0 of 14 (0%) cancers were GATA3 negative in the hormone-responsive group ($P = 0.031$) [22]. These results are in line with the current study, where we analyzed *GATA3* gene expression levels in 148 ER-positive recurrent breast cancer patients who were treated with first-line tamoxifen and found that high levels of *GATA3* were associated with a prolonged PFS (Fig. 2B). Moreover, in multivariate analysis, *GATA3* expression was an independent predictor of progression-free survival (Table 4). The predictive effect of GATA3 at the protein level, however, requires independent examination.

In the current study, we also observed that breast cancers with a *GATA3* mutation had significantly higher *GATA3* expression levels, although only *GATA3* expression appeared to be associated with prolonged PFS. Importantly, from the 76 breast cancers with high *GATA3* expression levels, 18 (23.7%) had a *GATA3* mutation. Thus, a significant fraction (n = 58, 76.3%) of the *GATA3* high breast cancers does not have a *GATA3* mutation. In order to be certain that we did not miss any mutations located outside exons 5 and 6, we additionally sequenced the other coding exons (*i.e.* exons 2–4) of the *GATA3* gene in these 58 breast cancers, but did not find any additional mutations. This not only confirms that the vast majority of *GATA3* mutations are actually located in exons 5 and 6, but this also suggests that there are other mechanisms besides *GATA3* mutation that may be responsible for the high *GATA3* expression in ER-positive breast cancers with a wild-type *GATA3* gene. As the GATA3 transcription factor reshapes gene loci by recruiting chromatin remodeling complexes, mutations of these proteins present in these complexes or other upstream pathway members could very well be involved in these mechanisms. For example, GATA3 expression was recently also reported to be increased by Wnt/β-catenin pathway activation in adipocytes [43]. Identification of these players may lead to a better understanding of the mechanisms of resistance to tamoxifen treatment by transcriptional regulation through GATA3, the crucial transcription factor regulating luminal differentiation in the mammary gland.

In conclusion, not *GATA3* mutation, but *GATA3* gene expression is associated with prolonged PFS in ER-positive breast cancer patients who received first-line tamoxifen treatment for recurrent disease. In addition, *GATA3* mutation leads to an increased *GATA3* mRNA expression, but besides genetic aberration of *GATA3*, other mechanisms are in place to explain the increased *GATA3* levels in *GATA3* wild-type tumors with high *GATA3* mRNA.

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Authors' contributions

Jingjing Liu contributed to the analysis and interpretation of data and wrote the manuscript. Wendy J.C. Prager–van der Smissen performed experiments and acquired data. Maxime P. Look and Marcel Smid contributed to the analysis and interpretation of the data. Anieta M. Sieuwerts contributed to study concept design, performed experiments and acquired data. Marion E. Meijer–van Gelder contributed to data acquisition. John A. Foekens and John W.M. Martens contributed to study concept design and wrote the manuscript. Antoinette Hollestelle contributed to study concept design, performed experiments and acquired data, contributed to analysis and interpretation of the data and wrote the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.03.038.

References

[1] A.H. Sims, A. Howell, S.J. Howell, R.B. Clarke, Origins of breast cancer subtypes and therapeutic implications, Nat. Clin. Pract. Oncol. 4 (2007) 516–525.
[2] S. Frkovic-Grazio, M. Bracko, Long term prognostic value of Nottingham histological grade and its components in early (pT1N0M0) breast carcinoma, J. Clin. Pathol. 55 (2002) 88–92.
[3] R. Clarke, J.J. Tyson, J.M. Dixon, Endocrine resistance in breast cancer – an overview and update, Mol. Cell. Endocrinol. 418 (2015) 220–234.
[4] D. Grote, A. Souabni, M. Busslinger, M. Bouchard, Pax 2/8-regulated Gata 3 expression is necessary for morphogenesis and guidance of the nephric duct in the developing kidney, Development 133 (2006) 53–61.
[5] C.K. Kaufman, P. Zhou, H.A. Pasolli, M. Rendl, D. Bolotin, K.C. Lim, et al., GATA-3: an unexpected regulator of cell lineage determination in skin, Genes Dev. 17 (2003) 2108–2122.
[6] M.L. Asselin-Labat, K.D. Sutherland, H. Barker, R. Thomas, M. Shackleton, N.C. Forrest, et al., GATA-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation, Nat. Cell Biol. 9 (2007) 201–209.
[7] H. Kouros-Mehr, E.M. Slorach, M.D. Sternlicht, Z. Werb, GATA-3 maintains the differentiation of the luminal cell fate in the mammary gland, Cell 127 (2006) 1041–1055.
[8] K.C. Lim, G. Lakshmanan, S.E. Crawford, Y. Gu, F. Grosveld, J.D. Engel, Gata3 loss leads to embryonic lethality due to noradrenaline deficiency of the sympathetic nervous system, Nat. Genet. 25 (2000) 209–212.
[9] R.V. Hoch, D.A. Thompson, R.J. Baker, R.J. Weigel, GATA-3 is expressed in association with estrogen receptor in breast cancer, Int. J. Cancer 84 (1999) 122–128.
[10] L.J. van't Veer, H. Dai, M.J. van de Vijver, Y.D. He, A.A. Hart, M. Mao, et al., Gene expression profiling predicts clinical outcome of breast cancer, Nature 415 (2002) 530–536.
[11] H. Kouros-Mehr, S.K. Bechis, E.M. Slorach, L.E. Littlepage, M. Egeblad, A.J. Ewald, et al., GATA-3 links tumor differentiation and dissemination in a luminal breast cancer model, Cancer Cell 13 (2008) 141–152.
[12] V. Theodorou, R. Stark, S. Menon, J.S. Carroll, GATA3 acts upstream of FOXA1 in mediating ESR1 binding by shaping enhancer accessibility, Genome Res. 23 (2013) 12–22.
[13] B.J. Wilson, V. Giguere, Meta-analysis of human cancer microarrays reveals GATA3 is integral to the estrogen receptor alpha pathway, Mol. Cancer 7 (2008) 49.
[14] R. Mehra, S. Varambally, L. Ding, R. Shen, M.S. Sabel, D. Ghosh, et al., Identification of GATA3 as a breast cancer prognostic marker by global gene expression meta-analysis, Cancer Res. 65 (2005) 11259–11264.
[15] N.K. Yoon, E.L. Maresh, D. Shen, Y. Elshimali, S. Apple, S. Horvath, et al., Higher levels of GATA3 predict better survival in women with breast cancer, Hum. Pathol. 41 (2010) 1794–1801.
[16] A. Albergaria, J. Paredes, B. Sousa, F. Milanezi, V. Carneiro, J. Bastos, et al., Expression of FOXA1 and GATA-3 in breast cancer: the prognostic significance in hormone receptor-negative tumours, Breast Cancer Res. 11 (2009) R40.
[17] N. Tominaga, Y. Naoi, K. Shimazu, T. Nakayama, N. Maruyama, A. Shimomura, et al., Clinicopathological analysis of GATA3-positive breast cancers with special reference to response to neoadjuvant chemotherapy, Ann. Oncol. 23 (2012) 3051–3057.
[18] H.E. Gulbahce, C. Sweeney, M. Surowiecka, D. Knapp, L. Varghese, C.K. Blair, Significance of GATA-3 expression in outcomes of patients with breast cancer who received systemic chemotherapy and/or hormonal therapy and clinicopathologic features of GATA-3-positive tumors, Hum. Pathol. 44 (2013) 2427–2431.
[19] T.K. Jenssen, W.P. Kuo, T. Stokke, E. Hovig, Associations between gene expressions in breast cancer and patient survival, Hum. Genet. 111 (2002) 411–420.
[20] V. Ciocca, C. Daskalakis, R.M. Ciocca, A. Ruiz-Orrico, J.P. Palazzo, The significance of GATA3 expression in breast cancer: a 10-year follow-up study, Hum. Pathol. 40 (2009) 489–495.
[21] M.A. Bollet, A. Savignoni, L. De Koning, C. Tran-Perennou, C. Barbaroux, A. Degeorges, et al., Tumor aromatase expression as a prognostic factor for local control in young breast cancer patients after breast-conserving treatment, Breast Cancer Res. 11 (2009) R54.
[22] P. Parikh, J.P. Palazzo, L.J. Rose, C. Daskalakis, R.J. Weigel, GATA-3 expression as a predictor of hormone response in breast cancer, J. Am. Coll. Surg. 200 (2005) 705–710.
[23] K.D. Yu, R. Zhu, M. Zhan, A.A. Rodriguez, W. Yang, S. Wong, et al., Identification of prognosis-relevant subgroups in patients with chemoresistant triple-negative breast cancer, Clin. Cancer Res. 19 (2013) 2723–2733.
[24] M.C. Labastie, D. Bories, C. Chabret, J.M. Gregoire, S. Chretien, P.H. Romeo, Structure and expression of the human GATA3 gene, Genomics 21 (1994) 1–6.
[25] H. Van Esch, P. Groenen, M.A. Nesbit, S. Schuffenhauer, P. Lichtner, G. Vanderlinden, et al., GATA3 haplo-insufficiency causes human HDR syndrome, Nature 406 (2000) 419–422.
[26] Cancer Genome Atlas Network, Comprehensive molecular portraits of human breast tumours, Nature 490 (2012) 61–70.
[27] S. Banerji, K. Cibulskis, C. Rangel-Escareno, K.K. Brown, S.L. Carter, A.M. Frederick, et al., Sequence analysis of mutations and translocations across breast cancer subtypes, Nature 486 (2012) 405–409.
[28] C. Kandoth, M.D. McLellan, F. Vandin, K. Ye, B. Niu, C. Lu, et al., Mutational landscape and significance across 12 major cancer types, Nature 502 (2013) 333–339.
[29] M.S. Lawrence, P. Stojanov, C.H. Mermel, J.T. Robinson, L.A. Garraway, T.R. Golub, et al., Discovery and saturation analysis of cancer genes across 21 tumour types, Nature 505 (2014) 495–501.
[30] K.U. Gaynor, I.V. Grigorieva, M.D. Allen, C.T. Esapa, R.A. Head, P. Gopinath, et al., GATA3 mutations found in breast cancers may be associated with aberrant nuclear localization, reduced transactivation and cell invasiveness, Horm. Cancer 4 (2013) 123–139.
[31] J. Usary, V. Llaca, G. Karaca, S. Presswala, M. Karaca, X. He, et al., Mutation of GATA3 in human breast tumors, Oncogene 23 (2004) 7669–7678.
[32] Y.Z. Jiang, K.D. Yu, W.J. Zuo, W.T. Peng, Z.M. Shao, GATA3 mutations define a unique subtype of luminal-like breast cancer with improved survival, Cancer 120 (2014) 1329–1337.
[33] M.J. Ellis, L. Ding, D. Shen, J. Luo, V.J. Suman, J.W. Wallis, et al., Whole-genome analysis informs breast cancer response to aromatase inhibition, Nature 486 (2012) 353–360.
[34] L.M. McShane, D.G. Altman, W. Sauerbrei, S.E. Taube, M. Gion, G.M. Clark, et al., Reporting recommendations for tumor marker prognostic studies (REMARK), J. Natl. Cancer Inst. 97 (2005) 1180–1184.
[35] A.M. Sieuwerts, M.E. Meijer-van Gelder, M. Timmermans, A.M. Trapman, R. Rodriguez-Garcia, M. Arnold, et al., How ADAM-9 and ADAM-11 differentially from estrogen receptor predict response to tamoxifen treatment in patients with recurrent breast cancer: a retrospective study, Clin. Cancer Res. 15 (2005) 7311–7321.
[36] J.A. Foekens, H. Portengen, W.L. van Putten, H.A. Peters, H.L. Krijnen, J. Alexieva-Figusch, et al., Prognostic value of estrogen and progesterone receptors measured by enzyme immunoassays in human breast tumor cytosols, Cancer Res. 49 (1989) 5823–5828.
[37] J.A. Foekens, H. Portengen, M.P. Look, W.L. van Putten, B. Thirion, M. Bontenbal, et al., Relationship of PS2 with response to tamoxifen therapy in patients with recurrent breast cancer, Br. J. Cancer 70 (1994) 1217–1223.
[38] T. van Agthoven, A.M. Sieuwerts, M.E. Meijer-van Gelder, M.P. Look, M. Smid, J. Veldscholte, et al., Relevance of breast cancer antiestrogen resistance genes in human breast cancer progression and tamoxifen resistance, J. Clin. Oncol. 27 (2009) 542–549.
[39] J.L. Hayward, P.P. Carbone, J.C. Heuson, S. Kumaoka, A. Segaloff, R.D. Rubens, Assessment of response to therapy in advanced breast cancer: a project of the Programme on Clinical Oncology of the International Union Against Cancer, Geneva, Switzerland, Cancer 39 (1977) 1289–1294.
[40] European Organization for Research and Treatment of Cancer, Breast Cancer Cooperative Group, Manual for Clinical Research and Treatment in Breast Cancer, Excerpta Medica, Almere, The Netherlands, 2000, pp. 116–117.
[41] M. Riaz, E.M. Berns, A.M. Sieuwerts, K. Ruigrok-Ritstier, V. de Weerd, A. Groenewoud, et al., Correlation of breast cancer susceptibility loci with patient characteristics, metastasis-free survival, and mRNA expression of the nearest genes, Breast Cancer Res. Treat. 133 (2012) 843–851.
[42] J.T. den Dunnen, S.E. Antonarakis, Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion, Hum. Mutat. 15 (2000) 7–12.
[43] L. Wang, L.J. Di, Wnt/β-Catenin mediates AICAR effect to increase GATA3 expression and inhibit adipogenesis, J. Biol. Chem. 290 (2015) 19458–19468.