High BRCA1 Gene Expression Increases the Risk of Early Onset Distant Metastasis in ER+ Breast Cancers

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3 **High *BRCA1* gene expression increases the risk of early onset**

4 **distant metastasis in ER⁺ breast cancers**

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27 **Abstract**

28 **Background:** More than 30% of ER⁺ breast cancer patients developed distant metastasis
29 after adjuvant tamoxifen therapy. It has been shown that decreased BRCA1 protein
30 function confers anti-estrogen “resistance.” Since the functional status of BRCA1 protein
31 auto-regulates its own gene expression, *BRCA1* transcript level should possibly be used
32 to assess its active protein levels. Thus, it is interesting to explore the potential links
33 between *BRCA1* gene expression status and the prognosis of ER⁺ breast tumors.

34 **Methods:** Early-staged ER⁺ Breast cancer microarray samples were selected from the
35 NCBI Gene Expression Omnibus (GEO) database. The aggressiveness of these tumors
36 was evaluated based on molecular subtypes or patients’ distant metastasis-free survival
37 (DMFS) time found in associated GEO datasets. In survival analysis, the optimal
38 threshold to define high and low transcript levels was assessed by the logistic-accelerated
39 failure time mixture regression model in a pooled data set, which simultaneously
40 envisages the lifetime risk of distant metastasis and the distribution of the DMFS time
41 from the surgery of a patient susceptible to distant metastasis.

Results: On the basis of molecular subtyping, the relatively aggressive Luminal B tumor cells expressed significantly more *BRCA1* transcripts than the less aggressive Luminal A tumor cells. This observation was supported by DMFS time analysis. When the upper 10% of the high *BRCA1* expression patients was compared with the rest of the patients, the result was quite drastic. In patients susceptible to distant metastasis, the median onset time of distant metastasis for high *BRCA1* expression group (2.2 years) was about one-fifth of the *BRCA1* low expression group (10.5 years).

Conclusions: High *BRCA1* transcript level does not increase the incidence of distant metastasis, but increases the risk of early onset distant metastasis in ER⁺ breast cancers. As new therapeutic drugs have been developed for tumors with BRCA function loss, our findings suggest that *BRCA1* gene expression test not only is useful for predicting patients' outcome but also can be used in treatment decision.

Key words: *BRCA1*, early breast cancer, distant metastasis-free survival, ER⁺, tamoxifen, cure model

56 **Introduction**

57 Breast cancer has been the most common and the leading cause of cancer deaths in
58 women worldwide [1,2]. Most breast cancers are sporadic and have no family history.
59 Based on their molecular characteristics [3], breast cancer can be classified into different
60 subtypes. These subtypes may help predicting patient outcome and serve as targets for
61 adjuvant therapies. According to the American Cancer Society, two out of three breast
62 cancers are positive for hormone receptors and most of them are estrogen receptor
63 positive (ER⁺). Binding to ER, estrogen may activate estrogen-ER signaling pathway and
64 promote breast tumor formation [4,5] or induce the proliferation of ER⁺ breast tumor
65 cells [6-8]. Therefore, a standard adjuvant therapy to treat ER⁺ breast cancers is to
66 suppress the estrogen-induced proliferation by anti-estrogen therapies, such as using
67 tamoxifen. However, despite the clinical success of tamoxifen, about 22-52% of early
68 ER⁺ breast tumors exhibit recurrence in long-term follow up studies after this
69 anti-estrogen treatment [9]. What causes this resistance is not clear yet, but decreased
70 Breast cancer type 1 susceptibility protein (BRCA1) has been reported to confer

71 tamoxifen resistance [10].

72 In addition to its well-known role in DNA damage repair [11], BRCA1 has many

73 important biological functions. One of them is to regulate mammary cell growth and

74 differentiation through interacting with estrogen-ER signaling pathway [12,13]. By

75 forming a complex with ER, BRCA1 induces ER conformation change and regulates

76 ER's transcriptional activity [12]. Loss of BRCA1 function increases ER-coactivator

77 association in tamoxifen treated cancer cells, which leads to tamoxifen resistance [10]. In

78 fact, decreased BRCA1 protein staining is commonly observed in sporadic breast cancers

79 [14,15] and this phenomenon is associated with high histologic grades [16-18] and short

80 disease-free survival [19]. It is certain that such association is related to an E-ER

81 signaling pathway.

82 If BRCA1 function does play a decisive role in breast cancer prognosis, this protein

83 should be a good predictor for patients' prognosis or even can serve as a target for

84 treatment. However, there are still many problems to be solved before this idea can be

85 applied clinically. One of them is how to develop a precise measuring tool to check the

86 active BRCA1 protein level in a tumor. Gene-expression based methods, such as PCR,
87 could be good candidates, readily quantifiable, reliable, and cost-effective.

88 When the BRCA1 protein loses its function, a cell may undergo more mutations due to
89 defective DNA repair and be transformed into a cancer cell. As BRCA1 protein
90 auto-regulates its own gene transcription [20], this loss-of-function mutation may lead to
91 a high *BRCA1* gene expression. Therefore, high *BRCA1* expression might be associated
92 with aggressive behavior in breast tumors. This assumption was supported by previous
93 study results showing that *BRCA1* mRNA level was up-regulated in tumors [21-23] and
94 that high *BRCA1* gene expression was associated with fast metastasis [24]. However,
95 some earlier studies presented opposite findings [25-28]. These controversial
96 observations need to be resolved before exploring the possibility of using a
97 gene-expression test to check active BRCA1 protein function.

98 This study used gene expression microarrays from the public domain to explore the
99 association between *BRCA1* mRNA level and the aggressiveness of breast tumors.

100 Molecular subtypes and distant metastasis-free survival (DMFS) were used to assess the

aggressiveness of tumors, respectively. Among all different molecular subtypes of breast cancers, only luminal A and B subtypes were selected to compare *BRCA1* mRNA levels because both subtypes are ER⁺. In survival analysis, only early-stage ER⁺ patients who received standard tamoxifen treatment after surgery were included in the DMFS analysis in attempt to attain homogeneity among study subjects. A “cure model” [29] was utilized in this study to tackle the non-susceptibility of distant metastasis, which would be ignored by the commonly-used Cox proportional hazards regression model [30] in survival analysis.

Materials and Methods

Study samples

Microarray data used in this study were all from the Gene Expression Omnibus (GEO) repository [31] located at the National Center for Biotechnology Information (NCBI). To examine *BRCA1* gene expressions in molecular subtypes of ER⁺ breast tumors, we chose dataset GSE45827 [32], which is composed of 130 primary invasive breast cancers (41 Triple Negative, 30 Her2, 29 Luminal A and 30 Luminal B tumors) as well as 11 normal

116 tissues and 14 cell lines using Affymetrix HG U133 plus 2.0 as the study platform.

117 Because this study focused on ER⁺ tumors, only the *BRCA1* expression levels of normal

118 breast tissue, Luminal A, and Luminal B tumors from GSE45827 were compared.

119 Two criteria were considered in selecting the datasets for survival analysis: the first was

120 to acquire datasets that tracked the survival time with related clinical data, and the second

121 was to use datasets with an identical technology platform for microarray gene expression

122 data. Ten breast cancer datasets with survival records, which used the U133A platform for

123 expression analysis, were thus selected from the GEO database (see clinical information

124 in Supplementary Table S1). However, because DMFS could be more directly associated

125 with tumor behavior, the DMFS time was used as the endpoint of patients' prognosis so

126 that only those datasets with the DMFS time were selected for our study. To make our

127 study samples homogenous, the following inclusion criteria were further used to select

128 ER⁺ patients for early detection and treatment: 1) the tumor was localized at its original

129 locus, 2) the lymph nodes were free of tumor, and 3) the tumor size was not larger than 5

130 cm. Study subjects must have an estrogen receptor status and adjuvant treatment

information. After thoroughly examining patients' clinical information, only 359 patients in three GEO datasets (GSE12093 [33], GSE17705 [34] and GSE45255 [35]) were entered into our survival analysis (see Supplementary Table S2).

Microarray data analysis

The microarray data were normalized by using the Bioconductor R "affy" package [36]. In order to minimize the false-negative and false-positive rates, the parameters were set up based on Choe's [37] study. Namely, `bgcorrect.method = "mas"`, `pmcorrect.method = "pmonly"`, `normalize.method = "quantiles"`, and `summary.method = "medianpolish"` were set up and input into the "affy" package's "expresso" function. All samples of GSE45827 (on HG-U133 plus 2.0 platform) were normalized. The 573 samples pooled from three chosen GEO datasets, GSE12093, GSE17705 and GSE45255 (on HG-U133A platform), were normalized together. After the normalization, eligible study samples satisfying the aforementioned inclusion criteria were selected for further analysis.

The R package "jetset" [38] was used to select the most representative probe set from

multiple probe sets of a gene in the Affymetrix microarray. This program computes “jetscores” and uses the probe set of the highest score for a gene designated for further analysis. In Affymetrix HG U133 series, the *BRCA1* gene has two corresponding probe sets, 204531_s_at and 211851_x_at with “jetscores” of 0.278 and 0.029, respectively. Therefore, 204531_s_at was substituted for *BRCA1* gene expression in our analysis.

151

152 **Statistical methods**

153 **The Kruskal-Wallis test**

To compare the differences in the *BRCA1* gene expression levels among several groups, the nonparametric Kruskal-Wallis test was used to test the null hypothesis that these independent samples came from the same population distribution.

157

158 **Logistic-accelerated failure time (AFT) mixture regression model**

We employed the logistic-AFT mixture regression model [29] to compare two

160 Kaplan-Meier curves [39] representing the DMFS time between high-level and low-level
 161 *BRCA1* expressions, which was defined by a specified cut-off point of *BRCA1*
 162 expression values, with only right censored data. A patient was either an event case with
 163 distant metastasis or right censored with a follow-up DMFS time. Fitting a patient's
 164 indicator covariate representing the high-*BRCA1* expression into the mixture model
 165 simultaneously estimates her lifetime risk probability of distant metastasis and the
 166 distribution of her DMFS time if she would be susceptible to distant metastasis. A brief
 167 introduction to the mixture model is as follows:

168 Let T be the duration in years from the surgery to distant metastasis in breast cancer, and
 169 the survival distribution at t years $\Pr(T > t)$ indicates the probability that a patient's cancer
 170 will not metastasized for t years. The patient's eventual susceptibility to distant metastasis
 171 is represented by the binary indicator D , $D = 1$ indicating the patient is susceptible, $D = 0$
 172 indicating the patient is not susceptible. The probability that a patient with a risk factor Z ,
 173 such as an indicator of high-*BRCA1* expression, will not metastasized after t years is
 174 expressed as:

175 $\Pr(T > t|Z) = \Pr(D = 1|Z)\Pr(T > t|D = 1, Z) + \Pr(D = 0|Z),$
 176 where, for a given Z , $\Pr(D = 0|Z)$ is the lifetime DMFS probability and $\Pr(T > t|D = 1, Z)$
 177 is the conditional survival function at t years for a susceptible patient.

178 The logistic-AFT mixture regression model [29] contains both a logistic regression
 179 submodel

$$180 \quad \ln\left(\frac{\Pr(D=1|Z)}{\Pr(D=0|Z)}\right) = \beta'Z,$$

181 and an AFT location-scale regression submodel

$$182 \quad \ln T(Z) = \gamma'Z + \exp(\alpha'Z)\varepsilon.$$

183 Parameters β , γ , and α are in the logistic, location, and scale regression parts,
 184 respectively, and can be simultaneously estimated using the R package
 185 “MixtureRegLTIC”[40]. For a specific value of Z , the logistic submodel provides a
 186 parametric estimator for the lifetime DMFS probability $\Pr(D = 0|Z)$, which can also be
 187 estimated nonparametrically by the tail of the Kaplan-Meier overall DMFS curve. In
 188 contrast, the AFT submodel formulates the parametric distribution of the DMFS time in
 189 the logarithm scale for a susceptible patient, while the Kaplan-Meier conditional DMFS
 190 curve is displayed as its nonparametric counterpart.

191 Since the log-logistic distribution was much better fitted to our pooled GSE breast cancer
192 datasets for the AFT submodel than the other generalized gamma distribution available in
193 the MixtureRegLTIC, we assumed that T of a susceptible patient follows a log-logistic
194 distribution in the AFT location-scale regression submodel; that is, ε is a logistic error
195 term with the density $f(\varepsilon) = e^\varepsilon / (1 + e^\varepsilon)^2$.

196 According to the minimum Akaike information criterion (AIC), we determined the best
197 model selection of the covariate to be included in the suitable regression parts of the
198 logistic-AFT location-scale mixture regression model. To check the adequacy of the fitted
199 model for high and low BRCA1 expressions, we plotted the overall survival curves $\Pr(T >$
200 $t|Z)$ and the conditional curves $\Pr(T > t|D = 1, Z)$ by superimposing on the corresponding
201 Kaplan-Meier overall and conditional curves to visualize the goodness-of-fit for the
202 best-fitted mixture model. The resultant p-value of the likelihood ratio test in the
203 best-fitted mixture model is used for evaluating the significance of the difference between
204 the Kaplan-Meier overall DMFS curves.

206

207 **Results**

208 **High *BRCA1* gene expression is associated with aggressive behavior in**

209 **ER⁺ breast tumors**

210 BRCA1 is known to regulate mammary cell proliferation through interacting with ER.

211 Thus, we focused on ER⁺ tumors (molecular subtypes luminal A and B) to make our

212 samples homogeneous. The GEO dataset GSE45827 contains Affymetrix HG-U133 plus

213 2.0 microarrays of normal breast tissues and primary breast tumors with different

214 molecular subtypes. It allows us to compare the *BRCA1* expression levels in different

215 molecular subtypes of ER⁺ tumors. In Figure 1, *BRCA1* expression levels in luminal B

216 tumors were significantly higher than those in normal breast tissues or luminal A tumors.

217 In contrast, *BRCA1* expression levels were not different in normal tissue and luminal A

218 tumors. Luminal A tumors are defined as tumors which are positive for ER but low in

219 either HER2 or proliferation marker Ki-67 levels. These tumors grow slowly and have a

220 good prognosis. Luminal B tumors are also ER⁺ but with a high level of proliferation

marker Ki-67. In other words, luminal B tumors grow faster than luminal A tumors. Our observation reveals that high *BRCA1* expression is associated with the proliferation of ER⁺ cells.

Figure 1. Boxplots of *BRCA1* expression in normal breast tissue, Luminal A and Luminal B tumors of GSE45827. Normal breast tissues (red), luminal A tumors (green) and luminal B tumors (blue) are displayed from left to right. Each dot represents the *BRCA1* expression level of a certain sample. Pairwise p-values between different subtypes were calculated using the Kruskal-Wallis test.

High *BRCA1* gene expression is associated with fast distant metastasis in ER⁺ early-stage breast cancers

Molecular subtyping clearly demonstrated the association between *BRCA1* gene

236 expression level and tumor aggressiveness. It is expected that this association can be
237 reflected in the patients' survival pattern. Thus, a pooled dataset with both microarray
238 data and the survival data were collected from the GEO repository. Since a patient may
239 not have a long-term survival once she develops a distant metastasis, the DMFS is a good
240 endpoint for the aggressiveness of tumors.

241 The *BRCA1* expression level is a continuous variable and the histogram, which is
242 composed of the *BRCA1* expression levels of the pooled samples from the three GEO
243 datasets, is unimodal. To compare the differences in the DMFS time of high- or
244 low-*BRCA1* expression groups, a threshold for *BRCA1* expression is needed. Different
245 deciles in the histogram of *BRCA1* expression distribution were used to categorize the
246 patients into high- or low-expression groups, respectively.

247 Results from fitting the logistic-AFT mixture model were not significant for each cut-off
248 point ranging from the 1st to 4th deciles (results not shown). Results falling into the 5th to
249 8th deciles were reported in Figure 2A-D and Table 1, and those for the cutoff-point at the
250 9th decile were displayed in Figure 3 and Table 2. In each of these figures, the left panel

251 indicates the overall DMFS curves and the right panel the conditional DMFS curves. The
252 former displayed the DMFS pattern for all patients, and the latter showed the DMFS
253 distribution of the patients who would be susceptible to distant metastasis. The time-axis
254 of the conditional DMFS curve was plotted in a logarithmic scale and could be used to
255 estimate the median onset time of distant metastasis for susceptible patients.

256 The difference between the Kaplan-Meier overall DMFS curves (step functions) for these
257 two expression groups defined by a specified decile was then examined. It was noted that
258 divided by higher deciles (such as 70% to 90%), the corresponding DMFS curves of the
259 high-*BRCA1* expression groups (the red step functions in the left panels of Figures 2C,
260 2D and 3) flatten off, respectively. In the high *BRCA1* expression group, some patients
261 developed distant metastasis early on around the first seven years after surgery. In contrast,
262 the rest (more than 60%) of the patients rarely metastasized in the 15-year follow-up. These
263 results implied that the hazard ratio of the high-expression group to the low one changed
264 over the follow-up course, so using the logistic-AFT mixture regression model [29] to fit
265 the pooled GEO data with *BRAC1*, instead of the Cox proportional hazards model [30],

266 was quite adequate.

267 The mixture regression model enabled us to predict the DMFS accurately so that the

268 resultant overall and conditional DMFS (smooth) curves defined at higher deciles derived

269 from the model fitting were much closer to the corresponding empirical Kaplan-Meier

270 overall and conditional DMFS curves (step functions). In addition to the significant

271 p-values resulting from the likelihood ratio test (LRT) for the mixture models (cut-off from

272 50% to 90%), a cutoff at 60 to 90 % (Tables 1 and 2) seems to emerge as a trend. The

273 Kaplan-Meier conditional DMFS curves for patients susceptible to distant metastasis (right

274 panels in Figure 2A to 2D and in Figure 3) found in the high- and low- *BRCA1* expression

275 groups were also separated more distinctly with increasing cut-off values. Our model

276 fitting obviously confirmed the *BRCA1*-gene expression dependency with increasing

277 *BRCA1*-expression levels. This trend was revealed with the statistical significance of the

278 high-*BRCA1* expression in the “Location Regression Part” in Tables 1 and 2, together

279 with the well-separated low- and high-*BRCA1* expression (smooth) curves in Figures 2

280 and 3. The separation due to a negative estimate of the location parameter resulted in a

281 smaller median onset time of distant metastasis in the high-*BRCA1* expression group than
282 in the lower one. On the other hand, each of the “Scale Regression Part” in Tables 1 and 2
283 did not include the high-*BRCA1* expression, so the shapes of the low- and high-*BRCA1*
284 expression conditional DMFS curves were similar.

285

286

287

Table 1. Analysis of the log-logistic-AFT mixture regression model stratified by BRCA1-level with different cut-off points.

Cut-off Decile	Covariate	Logistic Regression		AFT Submodel (Log-logistic Event Time Distribution)					Mixture Model p-value ^b
		Submodel		Location Regression Part			Scale Regression Part		
		Estimate	95% CI	Estimate	95% CI	p-value ^a	Estimate	95% CI	
50%	Intercept	-0.166	-1.773, 1.441	2.826	1.764, 3.888		-0.390	-0.739, -0.041	0.011
	BRCA1-H			-0.653	-1.163, -0.143	0.012			
60%	Intercept	-0.330	-1.780, 1.120	2.605	1.590, 3.620		-0.407	-0.764, -0.050	0.038
	BRCA1-H			-0.557	-1.088, -0.026	0.040			
70%	Intercept	-0.631	-1.505, 0.243	2.365	1.681, 3.049		-0.494	-0.823, -0.165	0.014
	BRCA1-H			-0.752	-1.332, -0.172	0.011			
80%	Intercept	-0.799	-1.414, -0.184	2.183	1.677, 2.689		-0.570	-0.866, -0.274	0.006
	BRCA1-H			-1.016	-1.659, -0.373	0.002			

Abbreviations: CI, confidence interval; BRCA1-H, high BRCA1; LRT, likelihood ratio test.

p-value^a of Wald's statistic for testing the location parameter corresponding to BRCA1-H.

p-value^b of the LRT for testing the significance of the fitted mixture model.

288

289

290 **Figure 2. Survival curves fitted by the “Logistic-AFT mixture regression model” at**

291 **the 5th-8th deciles.** Kaplan-Meier (step function) and the mixture regression (smooth

292 curve) estimators of overall and conditional DMFS curves for the distant metastasis onset

293 time after the surgery stratified by BRCA1-level defined at the cut-off point (A) 50th, (B)

294 60th, (C) 70th, and (D) 80th percentile, and corresponding p values of likelihood ratio test

295 (P_{LRT}) were 0.011, 0.038, 0.014 and 0.006, respectively. The *BRCA1* high- and

296 low-expression groups were shown in red and in blue, respectively, for each cut-off point.

297

298

299 Consequently, in Table 2, the p value of the LRT corresponding to the 9th decile was <0.001

300 (still significant even after adjustment for multiple comparison). It was the smallest among

301 the LRT p-values for all deciles; it suggested that the best-fitted model was based on the
302 90th percentile (of expression level 7.04) as the cut-off in the histogram for the pooled
303 *BRCA1* gene expression values. As shown in Figure 3, this cut-off point sharply contrasted
304 the difference for the two Kaplan-Meier DMFS curves depicting the high and low levels of
305 *BRCA1* expression. The decreasing trend of the AIC values (results not shown) among
306 increasing cut-off deciles implied that the possibility of over-fitting the model by using this
307 optimal cut-off point rather than other points was low.

308 The logistic regression submodel (Table 2) did not include the high-*BRCA1* expression
309 variable. The estimated intercept (-0.520) indicated that the estimated common lifetime
310 probability of the DMFS in both expression levels was 0.627. That is, 62.7% of the
311 early-stage ER⁺ tamoxifen-treated breast cancer patients were free of distant metastasis
312 during the 15 years of follow-ups, no matter what the *BRCA1* expression level was. It also
313 readily explained why the Kaplan-Meier overall DMFS curves depicting the *BRCA1* high-
314 (red step function) and low- (blue step function) expression groups in the left panel of
315 Figure 3 crossed at similar values of the DMFS proportion right before the 15th year of

316 follow-up. The location parameter in the AFT location-scale regression submodel,
317 however, included a high-*BRCA1* expression variable with a negative parameter estimate
318 -1.571 ($p < 0.001$ of the Wald test, Table 2). This indicated that *BRCA1* had an impact on
319 the distant metastasis after surgery time such that median onset time for susceptible
320 patients in the high-*BRCA1* expression group was significantly earlier than that of the low
321 expression group. The left panel of Figure 3 revealed that the Kaplan-Meier curve
322 illustrating the high *BRCA1* expression group (red step function) dropped sharply and then
323 flattened off afterwards. This was because distant metastasis in this group all occurred in
324 the first 7 years. Therefore, the rest of the patients appeared to be “cured” towards the end
325 of the 15-year follow-up period. Nevertheless, this phenomenon was not observed in the
326 low *BRCA1* expression group because patients with low *BRCA1* expression gradually
327 developed distant metastasis, *i.e.*, their relapses occurred in a steady rate during the 15
328 years. The median onset time of distant metastasis in the high- and low-expression groups
329 was estimated as 2.2 and 10.5 years, respectively. Because the median onset time of distant
330 metastasis for susceptible patients in the high *BRCA1* expression group was about one-fifth

(2.2 / 10.5 years) of that in the low expression group, these patients seemed to have extremely aggressive tumors. Therefore, *BRCA1* expression level may assist in identifying these high-risk patients. Patients in the high *BRCA1* expression group should be treated differently from other patients.

Figure 3. Survival curves fitted by the “Logistic-AFT mixture regression model” at the 9th decile. Kaplan-Meier (step function) and the mixture regression (smooth curve) estimators of overall and conditional DMFS curves for the distant metastasis onset time after the surgery stratified by *BRCA1*-level defined at the cut-off point 90th percentile. The *BRCA1* high- and low-expression groups were shown in red and in blue, respectively.

Table 2. Analysis of the logistic-AFT mixture regression model stratified by BRCA1-level cut-off at the 90th percentile.

	Covariates	Estimate	95% CI	p-value
Event Probability	Intercept	-0.520	-1.218, 0.178	0.144
Location	Intercept	2.351	1.836, 2.866	< 0.001
	BRCA1-H	-1.571	-2.263, -0.879	< 0.001
Scale	Intercept	-0.567	-0.841, -0.293	< 0.001
LRT mixture model				< 0.001

Abbreviations: CI, confidence interval; LRT, likelihood ratio test; BRCA1-H, high BRCA1.

Discussion

Tamoxifen is usually used to treat the ER⁺ patients. This drug is a selective estrogen receptor modulator (SERM), competes with estrogen for ER-binding and inhibits the proliferation stimulated by estrogen. It was reported that decreased BRCA1 expression reduced the cellular response to tamoxifen [10] through increased ER-coactivator association. As BRCA1 protein can auto-regulate its own gene transcription [20], a loss of BRCA1 protein function may activate gene transcription and lead to high-*BRCA1* gene levels.

In previous studies of the association between *BRCA1* gene expression level and tumor

behavior, conflicting observations came to light. Our results show that high *BRCA1* gene expression is associated with aggressive tumor behaviors and with fast distant metastasis in early stage ER⁺ breast cancers. Our findings may be contrasted with the survival study results reported by Hennigs et al. [41] from primary non-metastatic invasive breast cancer patients of different molecular subtypes, but we utilized survival data derived from a longer follow-up with additional *BRCA1* gene expression information. Our study still has some limitations. We had expected to acquire a sufficiently large sample size of study subjects from the public domain; however, constrained by different study designs, varied or unclear clinical information items, and inconsistent follow-up periods found in the GSE datasets, it was necessary to adopt the pooled-data approach instead of meta-analysis in this study. Furthermore, the decile value cut at 90 % indicated the best result, but the sample size of this high-*BRCA1* expression group was small. These factors might alter the statistical results, but the DMFS analysis illustrated by various cut-off points for the *BRCA1* expression groups provided with coherent results showing a clear trend.

371 The high *BRCA1* expression patients in this study displayed a biphasic survival curve,
372 which implied that these patients could be divided into two subgroups. The subgroup
373 susceptible to distant metastasis is likely to lose the BRCA1 protein function so that the
374 disseminated cells proliferated fast. In contrast, the non-susceptible subgroup may retain
375 a normal BRCA1 protein level. As a tumor suppressor, normal BRCA1 protein function
376 would lead to DMFS. In other words, these two types of high *BRCA1* expression patients
377 may have different outcomes.

378 It is also noted that about 60 % of patients in the pooled cohort were “cured” regardless
379 of the presence of high or low expression of the *BRCA1* gene. This observation indicated
380 that *BRCA1* expression status did not change the lifetime risk of DMFS. This cure
381 proportion may be attributable to the effect of breast surgery combined with adjuvant
382 tamoxifen therapy. Nevertheless, the time to distant metastasis was *BRCA1* expression
383 dependent. When the cutoffs were raised from 50% to 90%, the median onset times of
384 distant metastasis for the susceptible patients of high-*BRCA1* expression groups were
385 gradually shortened, while those of lower-expression groups consistently retained (Figure

386 2A to 2D and Figure 3). This observation implied that the onset time might be affected by
387 the proliferation rate of the disseminated tumors. Presumably, the higher the proliferation
388 rate, the earlier the onset time is.

389 In fact, 20% of sporadic breast tumors [14] did not show BRCA1 staining in either the
390 nucleus or cytoplasm, which demonstrated a defect in protein production. In some cases,
391 breast tumor cells showed cytoplasmic BRCA1 retention along with a loss of nuclear
392 staining [15], which implied a defect in nuclear transportation [42,43]. These BRCA1
393 protein-loss tumor cells were associated with poor prognosis and might induce high
394 *BRCA1* gene expression *via* auto-regulation.

395 Some tamoxifen resistant tumors retain responsiveness to estrogen and can be treated by
396 other types of anti-estrogens, such as aromatase inhibitors (AIs) [44]. However, AIs have
397 limitations because they can be only used for post-menopausal patients; therefore, for
398 recurrent tumors with BRCA1 function loss, poly (ADP-ribose) polymerase (PARP)
399 inhibitors might be a good alternative therapy. The PARP inhibitor was developed for
400 BRCA-mutated [45] tumors, but now researchers seek to expand its use to high-risk

401 patients with similar molecular characteristics [46-48]. BRCA1 protein is involved in the
402 homologous recombination repair pathway to repair DNA double strand breaks. With
403 BRCA1 defects, tumor cells are forced to use alternative DNA-repair pathways [49], such
404 as simple end joining, to fix DNA damage. By inhibiting alternative repair pathways,
405 PARP inhibitors lead tumor cells to die, because all DNA double strand break repair
406 capabilities for tumor cells were blocked. However, normal cells still retain a functional
407 homologous recombination repair pathway, so they can survive quite well upon receiving
408 PARP inhibitor treatment.

409 To develop a companion diagnostic for prescribing PARP inhibitors, the commonly used
410 immunohistochemistry (IHC) staining may not be the best choice since the IHC is
411 cumbersome and is not easy to standardize and automate. Furthermore, the specificity
412 and sensitivity of some IHC antibodies are questionable [50]. In contrast, gene
413 expression-based methods, such as polymerase chain reaction, are specific, sensitive, and
414 quantitative. The latter has the potential to identify which recurrent patients are
415 appropriate for PARP inhibitor therapy.

In summary, our study results can explain the conflicting results produced by previous studies. The high *BRCA1* gene expression may either favor or not favor the patients' survival. If the high *BRCA1* gene expression leads to a high expression of normal BRCA1 protein, the patients appear to be cured. If, however, the high *BRCA1* gene expression is caused by the loss of BRCA1 protein function, the patients are susceptible to fast distant metastasis.

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Declarations

Ethics approval and consent to participate

This work is a pooled analysis of public data from gene expression omnibus (GEO) at the

National Center for Biotechnology Information. There is no need to get ethics approval.

Consent for publication

All authors agreed for this submission.

Availability of data and material

This work only analyzed public data; no new data were generated.

Competing interests

566 All authors declared no competing interests.

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572 USA.

573 **Authors' contributions**

574 Hui-Ju Chang: conceived the idea, carried out the analysis, wrote the manuscript

575 Ueng-Cheng Yang: conceived the idea, revised the manuscript

576 Mei-Yu Lai: performed the cure model analysis

577 Chen-Hsin Chen: developed the logistic AFT cure model, revised the manuscript

578 Yang-Cheng Fann: helped with the programming, revised manuscript

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Figures

Figure 1

Boxplots of BRCA1 expression in normal breast tissue, Luminal A and Luminal B tumors of GSE45827. Normal breast tissues (red), luminal A tumors (green) and luminal B tumors (blue) are displayed from left to right. Each dot represents the BRCA1 expression level of a certain sample. Pairwise p-values between different subtypes were calculated using the Kruskal-Wallis test.

Figure 2

Survival curves fitted by the “Logistic-AFT mixture regression model” at the 5th-8th deciles. Kaplan-Meier (step function) and the mixture regression (smooth curve) estimators of overall and conditional DMFS curves for the distant metastasis onset time after the surgery stratified by BRCA1-level defined at the cut-off point (A) 50th, (B) 60th, (C) 70th, and (D) 80th percentile, and corresponding p values of likelihood ratio test (PLRT) were 0.011, 0.038, 0.014 and 0.006, respectively. The BRCA1 high- and low-expression groups were shown in red and in blue, respectively, for each cut-off point.

Figure 3

Survival curves fitted by the "Logistic-AFT mixture regression model" at the 9th decile. Kaplan-Meier (step function) and the mixture regression (smooth curve) estimators of overall and conditional DMFS curves for the distant metastasis onset time after the surgery stratified by BRCA1-level defined at the cut-off point 90th percentile. The BRCA1 high- and low-expression groups were shown in red and in blue, respectively.

Supplementary Files

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