PRECLINICAL STUDY

Co-amplification of *CCND1* and *EMSY* is associated with an adverse outcome in ER-positive tamoxifen-treated breast cancers

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Abstract Amplification of chromosome 11q13 is commonly seen in breast carcinomas and candidate genes from this region include *CCND1* and *EMSY*. Here, we investigate the prognostic significance of *CCND1* and *EMSY* amplification in a large series of breast carcinomas and in BRCA1 and BRCA2 mutation positive breast cancers. Amplification of *CCND1* and *EMSY* was assessed by fluorescent in situ hybridization. Both *CCND1* and *EMSY* amplifications were associated with a significantly worse outcome in ER-positive patients treated with tamoxifen only, in contrast to nonamplified tumors ($P = 8.55 \times 10^{-4}$ and $P = 8.35 \times 10^{-5}$, respectively). In multivariable Cox models, which included standard prognostic markers, co-amplification of *CCND1* and *EMSY* was significantly more predictive of outcome than was amplification of either gene alone or neither gene amplified in ER-positive tamoxifen-treated patients ($P = 5.47 \times 10^{-5}$). *EMSY* gene amplification was a significantly less common event in BRCA2 mutation carriers as compared to BRCA1 mutation carriers (9 versus 24%, respectively). In contrast, *CCND1* amplification occurred at a similar frequency in both BRCA1 and BRCA2 breast cancers (22 versus 18%, respectively). In summary, co-amplification of *CCND1* and *EMSY* identified a poor prognostic subset of ER-positive tamoxifen-treated patients. In addition, *EMSY* amplification occurred at a lower frequency in BRCA2 mutation carriers providing evidence to support *EMSY* amplification as a somatic surrogate for BRCA2 loss in sporadic breast cancer.

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Introduction

Gene amplification is a common mechanism leading to the overexpression of oncogenes in human cancers. Some genomic regions are frequently amplified in tumors; an example is chromosomal band 11q13. Amplification of this region, spanning over 7 Mb, is commonly seen in breast, head and neck, lung and bladder cancer [1, 2]. In breast cancer, four distinct regions of amplification have been identified and these regions may independently be amplified as small amplicons, or part of larger amplicons, resulting in co-amplification of multiple oncogenes [3–5].

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CCND1, the gene encoding for cyclin D1, has been the most studied oncogene on 11q13 and has long been thought of as a potential driver of this amplification event in breast cancer [5, 6]. Cyclin D1, an important regulator of the cell cycle, [7, 8] has been found amplified in 15–20% of breast cancers [4, 9, 10] and overexpressed in up to 80% of tumors [1, 5, 11, 12]. Amplification of *CCND1* has also been associated with a poor prognosis in estrogen receptor positive breast cancers [13–15]. One possible mechanism by which overexpression of cyclin D1 could lead to a poor prognosis is by conferring resistance to endocrine therapy such as tamoxifen. A recent study has provided experimental evidence that cyclin D1 is required for tamoxifen-induced cell proliferation and growth of tamoxifen-resistant cells in vitro [16]. In addition, analysis of *CCND1* amplification in a series of breast cancers randomized to 2 years of adjuvant tamoxifen or no treatment showed that *CCND1* gene amplification was a strong independent predictor of tamoxifen response [17]. These results suggest that amplification of *CCND1* may be a marker for identifying tumors in which tamoxifen may be having an agonistic effect.

Another candidate oncogene on 11q13 is p-21-activated kinase 1 (*PAK1*), a serine/threonine kinase, that has been found to regulate anchorage-independent growth, invasiveness and the abnormal organization of mitotic spindles of human breast cancer cells [18]. Amplification of *PAK1* has been reported in breast [3] and ovarian cancers [19–21]. *PAK1* has been shown to activate estrogen receptor alpha (ERα) by phosphorylation, leading to the upregulation of ER-regulated genes such as *CCND1* [22]. In breast cancer, amplification of *PAK1* has also been recently shown to be a predictor of tamoxifen resistance in ER-positive patients [23].

Chromosome 11q13 is a gene dense region and in addition to *CCND1* and *PAK1* several other genes have been implicated in this amplification event. One such gene is *EMSY*, a novel BRCA2 interacting protein, that has been found amplified in both breast [24] and ovarian cancers [20, 21]. Given the potential role for both *CCND1* and *PAK1* as predictors of tamoxifen response, we investigated the clinical significance of *EMSY* and *CCND1* amplification in a large cohort of clinically annotated breast cancers. In addition, we also evaluated amplification of *EMSY* and *CCND1* in breast carcinomas associated with known mutations in BRCA1 and BRCA2.

Materials and methods

Breast cancer TMA

The study cohort included 4,046 female patients with newly diagnosed, invasive breast cancer in British Columbia. The clinicopathological characteristics and the treatment strategies of the patients included in this study have previously been reported [25]. The median follow-up was 12.5 years and age at diagnosis was 60 years. All patients had been referred to the British Columbia Cancer Agency and staging, pathology, treatment and follow-up information were available. During the study era, 75% of breast cancer cases in the province were referred; nonreferred patients were generally elderly or treated by mastectomy without indications for adjuvant therapy. Abstracted clinical information included age, histology, grade, tumor size, number of involved axillary nodes, lymphatic or vascular invasion (LVI), ER status, type of local and initial adjuvant systemic therapy (AST), dates of diagnosis and first local, regional or distant recurrence and death. Representative areas of invasive carcinoma were selected and marked on the hematoxylin and eosin slides, and its corresponding tissue block to be sampled for the tissue microarray (TMA). A single core TMA was then assembled as previously described [20]. Fluorescent in situ hybridization (FISH) analysis was performed on 6 μm paraffin TMA sections.

FISH on TMAs

The *EMSY* probe was created from DNA isolated from the PAC clones B4, DJ18D12, and DJ85A11 (all PCR verified for exons 2 or 8 of *EMSY*) and was directly labeled with Spectrum Green by nick translation (Vysis, Downer's Grove, Illinois). The *EMSY* probe was then combined with the commercially available Spectrum Aqua labeled centromeric probe, CEP11 (Vysis, Downer's Grove, Illinois) and Spectrum Orange labeled *CCND1* probe (Vysis, Downer's Grove, Illinois). The FISH assay was performed as previously described [20]. The slides were co-denatured for 5 min at 73°C and hybridized for 18 h at 37°C on a HYBrite (Vysis, Downers Grove, Illinois). Posthybridization washes were done as previously described [20]. Slides were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Automated analysis of FISH signals was performed using Metasystems™ automated image acquisition and analysis system, Metafer (Metasystems, Altlussheim, Germany). The average gene copy number for each probe was calculated and the amplification ratio was determined. The amplification ratio was calculated as a ratio between the average copy per tumor cell for each gene and the average copy number for centromere 11. An amplification ratio ≥ 1.5 was considered amplified. This definition of amplification has been used in several other studies and was used for this current study [20, 21, 24].

BRCA1, BRCA2 and non-BRCA1/2 familial breast cancers

The collection of these paraffin embedded breast cancers was a collaborative effort between Vancouver General

Hospital (Vancouver, BC), Memorial University (St. John's, Newfoundland), McGill University (Montreal, Quebec), Spanish National Cancer Research Centre (Madrid, Spain) and Lund University Hospital (Lund, Sweden). FISH for *EMSY* and *CCND1* were successfully analyzed in 63 BRCA1 mutation carriers and 55 BRCA2 mutation carriers.

Results

Amplification of *CCND1* and *EMSY* in a population-based cohort of breast carcinomas

To evaluate the amplification of *CCND1* and *EMSY*, a three-color FISH assay was developed that included centromere 11 as a reference probe. FISH signals were successfully scored in 1,318 cases for both *CCND1* and *EMSY*. There was no statistically significant difference in survival between the cases for which results were obtained and those cases with missing data ($P = 0.088$). Overall for the entire cohort, *CCND1* was amplified in 397 (30%) of 1,318 cases, and *EMSY* was amplified in 191 (14.5%) of 1,318 cases. Amplification of *CCND1* correlated significantly with *EMSY* amplification ($P = 5.416 \times 10^{-44}$), positive-ER status ($P = 2.62 \times 10^{-5}$) and Ki67 ($P = 3.10 \times 10^{-4}$). In addition, *CCND1* amplification showed an inverse correlation with the basal subtype of breast cancer ($P = 0.01$). *EMSY* amplification showed only a significant correlation with *CCND1* amplification ($P = 5.416 \times 10^{-44}$).

Amplification of *CCND1* and *EMSY* and disease-specific survival (DSS)

When DSS was analyzed for patients with amplification of *CCND1* and *EMSY*, associations with several clinicopathological variables were observed (Table 1). For all patients, amplification of *CCND1* and *EMSY* identified a subset of patients associated with a poor prognosis compared to those patients without amplification (Fig. 1a, b). We were then interested in determining whether those patients with co-amplification of *CCND1* and *EMSY* had a poorer outcome as compared to those patients with either *CCND1* or *EMSY* amplified alone or neither amplified. As seen in Fig. 1c, those patients with co-amplification of both *CCND1* and *EMSY* were associated with significantly poorer outcome versus neither gene amplified (log rank P value $= 2.78 \times 10^{-4}$) or versus either *CCND1* or *EMSY* amplified (log rank P value 6.91×10^{-3}).

Cox regression analysis was performed to estimate the hazard ratio for *CCND1*-amplified cases adjusted for standard clinicopathological variables including age at diagnosis, grade, tumor size, nodal status and Her2 status. *CCND1* amplification remained a significantly independent prognostic variable in this model (P value $= 0.017$) (Supplementary Table 2). The same Cox regression analysis was performed for *EMSY* amplified cases, and *EMSY* amplification also remained a significant independent prognostic predictor in this model (P value $= 1.53 \times 10^{-3}$) (Supplementary Table 3). To investigate whether co-amplification of both *CCND1* and *EMSY* were of greater prognostic value

Table 1 Disease-specific survival in *CCND1*- and *EMSY*-amplified patients determined by Cox univariate analysis

| Clinicopathological variables	CCND1 amplification		EMSY amplification	
	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
All patients	1.312 (1.069–1.610)	0.009	1.423 (1.106–1.830)	0.006
Nodal status				
Node negative	0.978 (0.693–1.381)	0.901	1.248 (0.819–1.903)	0.302
Node positive	1.657 (1.281–2.143)	0.0001	1.553 (1.130–2.133)	0.007
ER status				
ER negative	1.359 (1.069–1.729)	0.012	1.603 (1.198–2.145)	0.001
ER positive	1.388 (0.925–2.083)	0.114	1.091 (0.654–1.818)	0.739
Treatment				
ER+, Tamoxifen only	1.635 (1.139–2.347)	0.008	1.986 (1.329–2.968)	0.001
No AST	0.930 (0.646–1.427)	0.841	1.085 (0.641–1.836)	0.762
Breast cancer subtypes				
Luminal (ER+, PR+, Her2−)	1.338 (1.030–1.737)	0.029	1.507 (1.096–2.073)	0.012
Luminal/Her+	0.983 (0.541–1.786)	0.955	2.099 (1.007–4.376)	0.048
Her2+ (Her2+, ER−, PR−)	1.588 (0.844–2.988)	0.152	0.991 (0.420–2.343)	0.984
Basal (ER−, PR−, Her2−) and (CK5/6+ or EGFR+)	1.290 (0.640–2.601)	0.476	1.178 (0.527–2.635)	0.690
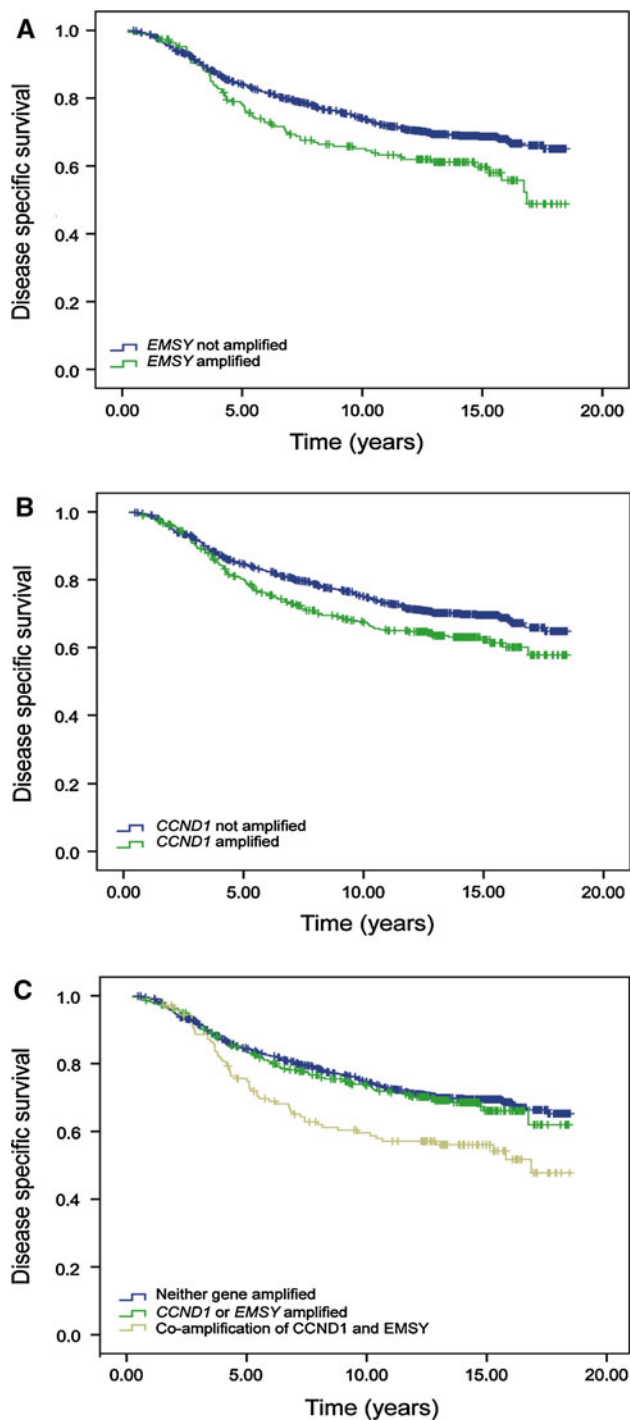
Fig. 1 DSS curve for **a** *EMSY* amplification in the entire cohort, **b** *CCND1* amplification in the entire cohort and **c** co-amplification of *CCND1* and *EMSY* in the entire cohort

than amplification of either gene alone, another Cox regression analysis was performed. The univariable hazard ratios for each covariate in the model can be seen in Table 2. Co-amplification of *CCND1* and *EMSY* remained a significantly independent prognostic variable in this model (P value = 4.62×10^{-4}).

Prognostic value of *CCND1* and *EMSY* within the tamoxifen-treated subset

In British Columbia, patients are treated according to provincial guidelines developed by the BC Cancer Agency. To investigate the prognostic value of *CCND1* and *EMSY* amplifications among ER-positive patients treated with tamoxifen only, and the additional effect of co-amplification of both genes, survival estimates were obtained for these subsets.

As seen in Table 1, those patients with amplification of *CCND1* and *EMSY* were associated with a significantly poor outcome in ER-positive tamoxifen-treated patients (P = 0.008 and P = 0.001, respectively). We next determined whether those patients with co-amplification of *CCND1* and *EMSY* were associated with a more adverse outcome in this subset of patients. Those patients with co-amplification of both genes were associated with a significantly adverse outcome in ER-positive tamoxifen-treated patients as compared to neither gene amplified (log rank P value = 5.6×10^{-5}) or versus either gene amplified (long rank P value = 3.69×10^{-3}) (Fig. 2a).

Cox regression analyses showed that amplification of both *CCND1* and *EMSY* was of independent prognostic value in ER-positive tamoxifen-treated patients (P values = 8.55×10^{-4} and 8.35×10^{-5}, respectively) (Supplementary Tables 4, 5). A third Cox model, when adjusted for standard clinicopathological variables, showed that co-amplification of both *CCND1* and *EMSY* were associated with a significantly worse survival (P = 3.91×10^{-6}) with a hazard ratio of 2.9 versus neither gene amplified (Table 3). In this subset of patients, co-amplification of both genes was the most significant prognosticator ahead of nodal status, grade and tumor size.

To further strengthen this finding, we also evaluated the prognostic significance of *CCND1* and *EMSY* amplification within the subset patients who received no adjuvant systemic therapy (AST). No statistically significant difference in survival was seen in those patients with co-amplification of both genes versus neither gene amplified (log rank P value = 0.302) (Fig. 2b).

Amplification of *CCND1* and *EMSY* in known BRCA1 and BRCA2 mutation carriers

We hypothesized that *EMSY* gene amplification would be a rare event in BRCA2 mutation carriers (as these cancers are already associated with a loss of BRCA2 function) and would be a frequent event in BRCA1 mutations carriers and familial breast cancers not associated with loss of either BRCA1 or BRCA2. To investigate this hypothesis, the same three-color FISH assay described above was performed on breast cancers associated with known BRCA1 and BRCA2

Table 2 Multivariable Cox proportional hazard models for age, grade, tumor size, nodal status, Her2 status, *CCND1* and *EMSY* gene status in 1,318 breast cancers

Clinicopathological variables	Hazard ratio (95% CI)	P value
Age, years		0.290
40–49 versus ≤40	0.763 (0.531–1.097)	0.144
50–65 versus ≤40	0.959 (0.679–1.353)	0.811
>65 versus ≤40	0.967 (0.676–1.382)	0.853
Grade		2.08×10^{-4}
Grade 2 versus grade 1	1.758 (0.714–4.330)	0.220
Grade 3 versus grade 1	2.770 (1.129–6.794	0.026
Tumor size		2.62×10^{-9}
2–5 cm versus ≤2 cm	1.627 (1.297–2.042)	2.62×10^{-5}
≥5 cm ≤2 cm	3.125 (2.152–4.539)	2.18×10^{-9}
Nodal status		
Node positive versus node negative	2.294 (1.844–2.854)	9.16×10^{-14}
Her2		
Her2 positive versus Her2 negative	1.401 (1.088–1.804)	0.009
CCND1 and EMSY		4.62×10^{-4}
CCND1 +ve and EMSY +ve versus neither amplified	1.817 (1.363–2.422)	4.63×10^{-5}
CCND1 +ve/EMSY −ve versus neither amplified	1.024 (0.777–1.349)	0.867
EMSY +ve/CCND1 −ve versus neither amplified	0.811 (0.447–1.515)	0.531

mutations. *CCND1* was also included in this analysis as a control as, we expected to see similar levels of *CCND1* amplification in all three subsets of familial breast cancer.

EMSY gene amplification was a frequent event in BRCA1 mutation carriers as expected (Table 4). In comparison, *EMSY* gene amplification was a significantly less common event in BRCA2 mutation carriers as compared to BRCA1 cases ($P = 0.047$). *CCND1* amplification was a frequent event in both BRCA1 and BRCA2 breast cancers (Table 4).

Discussion

This is the first study to analyze both amplification of *CCDN1* and *EMSY* in a large clinically annotated series of breast cancers. We found *CCND1* and *EMSY* to be amplified in 30 and 15% of breast cancers, respectively. In addition, we also determined that patients with co-amplification of *CCND1* and *EMSY* were associated with a significantly adverse outcome in ER-positive tamoxifen-treated patients. The findings were further strengthened in multivariate analyses, showing a ~threefold higher risk of dying of disease in tumors that were associated with co-amplification of *CCND1* and *EMSY*. In this subset of patients, co-amplification of *CCND1* and *EMSY* was the most significant prognosticator ahead of nodal status, tumor size and grade. These findings suggest that amplification of both *CCND1* and *EMSY* may be a predictor of tamoxifen resistance in breast cancer.

Amplification of *CCND1* has been well established in breast cancer, occurring at a frequency of 15–20%. *CCND1* amplification has also been associated with a poor prognosis in estrogen receptor positive breast cancers [13–15]. In line with these studies, we have found a similar, although slightly higher, frequency of *CCND1* amplification in our present study. One reason for this discrepancy could be the use of different cutoffs or methods used to determine gene amplification. However, all of these studies do confirm that *CCND1* is a frequent target of gene amplification in breast cancer. We also confirm in the present study that amplification of *CCND1* is associated with a poor prognosis in ER-positive breast cancers and in ER-positive tamoxifen-treated breast cancers as previously reported [17]. Amplification of *EMSY*, located ~6 Mb telomeric to *CCND1*, has also been commonly found amplified in breast cancer [24, 26]. The frequency of *EMSY* amplification found in this study is consistent with our previous findings [24]. Our findings are also consistent with a recent report, which found that *EMSY* amplification was associated with a decreased survival in ER-positive breast cancers [27].

Experimental studies have provided evidence to support a role for both *CCND1* and *PAK1* in tamoxifen response [16, 17, 23, 28]. It has been suggested that therapies targeting either *CCND1* or *PAK1* expression and/or activity may represent a novel strategy to increase endocrine response in breast cancer. Both *CCND1* and *PAK1* encode proteins that have been shown to activate ER [28], and increased expression of *PAK1* has been shown to stimulate
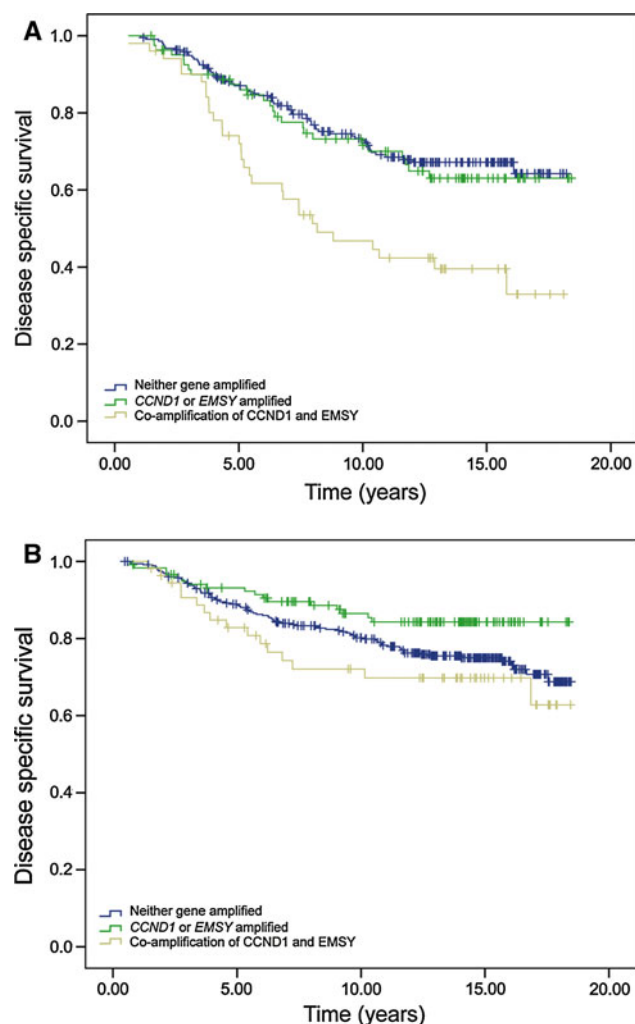
Fig. 2 DSS curve for **a** co-amplification of *CCND1* and *EMSY* in ER-positive tamoxifen-treated patients and **b** co-amplification of *CCND1* and *EMSY* within the subset of patients who received no adjuvant systemic therapy

Table 4 Amplification of *CCND1* and *EMSY* in known BRCA1 and BRCA2 mutation positive breast cancers

BRCA status	n	*CCND1* amplification	*EMSY* amplification
BRCA1	63	12 (22%)	15 (24%)
BRCA2	55	10 (18%)	5 (9%)

expression of *CCND1*. A recent study evaluating amplification of *CCND1* and *PAK1* in postmenopausal breast cancer patients randomized to tamoxifen treatment or no adjuvant therapy showed *PAK1* amplification to be a better predictor of tamoxifen resistance than amplification of *CCND1* [23].

The region of 11q13 contains numerous potential oncogenes, including *CCND1*, *EMSY*, *PAK1*, *Rsf-1* and many others. We have recently demonstrated that the 11q13 amplicon is frequently amplified in ovarian cancer and is likely driven by a cassette of genes rather than by a single oncogene [21]. This is likely true for breast cancer, although different genes may be involved. In this current study, we have shown that *CCND1* and *EMSY*, located less than 1 Mb centromeric to *PAK1*, are frequently amplified in breast cancer. Amplification of either gene alone is a strong independent prognostic indicator in both ER-positive breast cancers and in ER-positive tamoxifen-treated breast cancers. However, this effect on survival is even more pronounced when the two genes are co-amplified together. *EMSY* is a putative oncogene encoding a protein that interacts with the transactivation domain of BRCA2, and overexpression of a 5′ fragment of EMSY was found to induce chromosomal instability in normal human breast epithelial cells similar to that reported in BRCA2-deficient cells [21]. A role for *EMSY* in ER regulation has not been established. However, it is likely that amplification of

Table 3 Multivariable Cox proportional hazard models for age, grade, tumor size, nodal status, CCND1 and EMSY gene status in ER-positive tamoxifen-treated breast cancers

Clinicopathological variables	Hazard ratio (95% CI)	*P* value
Age, years		
≥50	0.971 (0.305–3.087)	0.960
Grade		
Grades 1,2 versus grade 3	2.096 (1.387–3.167)	4.46×10^{-4}
Tumor size		
>2 cm	2.287 (1.473–3.550)	2.3×10^{-4}
Nodal status		
Node positive versus node negative	2.694 (1.669–4.348)	4.94×10^{-5}
CCND1 and EMSY		5.47×10^{-5}
CCND1 +ve and EMSY +ve versus neither amplified	2.904 (1.847–4.565)	3.91×10^{-6}
CCND1 +ve/EMSY −ve versus neither amplified	1.257 (0.748–2.113)	0.387
EMSY +ve/CCND1 −ve versus neither amplified	0.741 (0.180–3.047)	0.678

several genes along the 11q13 amplicon may act in concert to drive amplification of this locus in breast cancer.

Lastly, we wanted to evaluate *EMSY* gene amplification in familial breast cancers associated with known mutations in BRCA1 and BRCA2. *EMSY* has been shown to interact with the transactivation domain of BRCA2. Mutations in BRCA1 and BRCA2 are rare events in sporadic breast cancer [29–31]. Although epigenetic silencing of BRCA1 has been reported in some sporadic cancers [32–36], no such silencing has been reported for BRCA2 [37–39]. It has been hypothesized that *EMSY* amplification may be a surrogate for BRCA2 loss in sporadic cancer [40, 41]. Therefore, mutations in BRCA2 may be unnecessary when *EMSY* is amplified and overexpressed. Indeed, we found that *EMSY* was less commonly amplified in BRCA2 mutation carriers (i.e., those cancers associated with loss of BRCA2 function) as compared to BRCA1 mutations carriers. Further evidence to support this association between *EMSY* and BRCA2 mutation carriers came from the parallel study of *CCND1* gene amplification in these same subsets of familial breast cancers. Amplification of *CCND1* was commonly observed at a similar frequency in BRCA1 and BRCA2 mutation carriers. Two studies have evaluated *CCND1* gene amplification in BRCA1 mutation carriers, although they both yielded discordant results. One study reported that *CCND1* was not amplified in 30 BRCA1 carcinomas [42], whereas another study reported *CCND1* amplification in 2 of 11 (18%) cases [43]. The latter study found a frequency of amplification very similar to what we observed, although the number of BRCA1 cases was much smaller than the 63 cases of BRCA1 carcinomas we analyzed.

In summary, these results demonstrate that co-amplification of *CCND1* and *EMSY* is associated with a poor prognosis in ER-positive tamoxifen-treated patients, and in multivariate analysis co-amplification of both genes was a very strong independent prognosticator. These findings suggest that in addition to *CCND1* and *PAK1*, other genes commonly amplified on 11q13 like *EMSY* may also be a potential predictor of tamoxifen resistance in breast cancer. Larger clinical studies of patients randomized to tamoxifen treatment or no adjuvant therapy are needed to map the cassette of genes involved in this amplicon. It may be that the 11q13 amplicon in breast cancers is responsible for driving tumorigenesis through the activation of several oncogenes that provide a selective advantage for tumors cells that are resistant to the effects of tamoxifen. Lastly, we found *EMSY* gene amplification to be a less common event in BRCA2 mutation carriers as compared to BRCA1 mutation carriers, providing evidence to support the potential role of *EMSY* as a surrogate for BRCA2 loss in sporadic breast cancer.

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