Adverse Effect of Adjuvant Tamoxifen in Premenopausal Breast Cancer with *Cyclin D1* Gene Amplification

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Abstract

Cyclins D1 and A2 are cell cycle regulators that also have the ability to interact with the estrogen receptor (ER) and consequently interfere with antiestrogen treatment in breast cancer. Experimental data support this concept, but the clinical relevance needs to be further established. In this study, we evaluated cyclin D1 and A2 protein expression by immunohistochemistry and cyclin D1 gene (*CCND1*) amplification by fluorescence *in situ* hybridization in 500 primary breast cancers arranged in tissue microarrays. Patients had been randomized to 2 years of adjuvant tamoxifen or no treatment with a median follow-up of 14 years, allowing for subgroup analysis of treatment response defined by cyclin status. We found that both cyclin D1 and A2 protein overexpression was associated with an impaired tamoxifen response, although not significant in multivariate interaction analyses, whereas tamoxifen-treated patients with CCND1-amplified tumors had a substantially increased risk for disease recurrence after tamoxifen treatment in univariate analyses [relative risk (RR), 2.22; 95% confidence interval (95% CI), 0.94-5.26; *P* = 0.06] in contrast to nonamplified tumors (RR, 0.39; 95% CI, 0.23-0.65; *P* < 0.0001). Consequently, a highly significant interaction between tamoxifen treatment and CCND1 amplification could be shown regarding both recurrence-free survival (RR, 6.38; 95% CI, 2.29-17.78; *P* < 0.001) and overall survival (RR, 5.34; 95% CI, 1.84-15.51; *P* = 0.002), suggesting an agonistic effect of tamoxifen in ER-positive tumors. In node-positive patients, the disparate outcome according to gene amplification status was even more accentuated. In summary, our data implicate that despite a significant correlation to cyclin D1 protein expression, amplification status of the *CCND1* gene seems a strong independent predictor of tamoxifen response, and possibly agonism, in premenopausal breast cancer. (Cancer Res 2005; 65(17): 8009-16)

Introduction

Loss of normal growth control, including aberrant cell cycle regulation, is one of the hallmarks of cancer (1). Central in the regulation of the G_1-S transition in the cell cycle is the p16/cyclin D/retinoblastoma protein (pRb) pathway that seems deregulated in a large fraction of all malignancies (2). In breast cancer, overexpression of cyclin D1 is observed in up to 50% of primary breast cancers with amplification of its corresponding gene *CCND1* in about 15% (3–6). Non-cell cycle–associated functions have also been shown for cyclin D1, such as binding to the estrogen receptor (ER) together with the cofactor SRC-1, potentially activating the receptor in a ligand-independent fashion (7, 8).

Whereas cyclin D1 is essential for G_1 progression, cyclin A2 functions during S phase and at the G_2-M transition. Cyclin A2 may, similarly to cyclin D1, also effect the ER and via activation of cyclin-dependent kinase 2 induce phosphorylation of $Ser^{104/106}$ of the ER, potentially inducing ligand-independent activation (9, 10). Thus, besides central roles in cell cycle regulation, cyclins D1 and A2 seem able to directly influence the ER and potentially modify its response to estrogens and antiestrogens. Selective ER modulators, with tamoxifen as the prototype, are the treatment of choice for hormone-dependent breast cancer. Today, the majority of all ER-positive breast cancer will receive adjuvant antiestrogen treatment and tamoxifen substantially improves patient survival (11). Nevertheless, it is also clear that a large fraction of patients do not respond to tamoxifen, despite having ER-positive tumors and some patients may even have an adverse outcome, due to the potential ER-agonistic effect of tamoxifen under certain conditions (12).

In line with a direct interaction of cyclins D1 and A2 on the ER, these proteins may be involved in tamoxifen resistance in breast cancer. A reduction in cyclin D1 expression seems an early and critical event in antiestrogen action *in vitro* (13, 14) and ectopic cyclin D1 expression in ER-positive cell lines blocks the antiestrogen effect (15). Gene expression analyses show that cyclin A2 is induced in response to estrogen as well as tamoxifen treatment, whereas cyclin D1 is constitutively expressed in tamoxifen-resistant cells (16). In one study, high cyclin A2 expression has been associated with an impaired tamoxifen response (17), but the predictive value has not yet been investigated in a randomized trial. We have previously shown an impaired tamoxifen response in postmenopausal women with cyclin D1–overexpressing breast cancer, in a randomized trial with long-term follow-up (18). Interestingly, the intensity of the nuclear staining by immunohistochemistry rather than the nuclear fraction was indicative of treatment response. The reason for this discrepancy remains to be elucidated, but the nuclear intensity of cyclin D1 might be linked to the degree of amplification of the *CCND1* gene (19).

In an attempt to define subgroups of breast cancer that respond differently to tamoxifen treatment, we therefore evaluated cyclin D1 and A2 expression in tissue microarrays (TMA) with primary breast cancer specimens from 500 premenopausal women included in a randomized trial with long-term follow-up. By comparing

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untreated patients with patients receiving tamoxifen, subgroups responding differently could be characterized. In addition, *CCND1* gene amplification was evaluated by fluorescence *in situ* hybridization (FISH) to explore the relationship with the staining intensity of the cyclin D1 protein and also to investigate whether the presence or absence of gene amplification had an independent treatment-predictive value.

Materials and Methods

Patient material. During 1984 to 1991, 564 premenopausal patients or patients under 50 years with stage II (pT2 N0 M0, pT1 N1 M0, and pT2, N1 M0) invasive breast cancer were enrolled in a randomized trial of 2 years of adjuvant tamoxifen treatment with a daily dosage of either 40 mg (study center 1) or 20 mg (study center 2) or no adjuvant treatment. Similar results for these dosages have been shown in several studies (20–22). Less than 2% of the patients (n = 9) received adjuvant polychemotherapy. The median follow-up for patients without breast cancer event was 13.9 years [95% confidence interval (95% CI), 13.6-14.3]. The study design is described in detail elsewhere (23).

Tissue microarray construction. Paraffin-embedded specimens from 500 cases could be retrieved from the archives. Representative areas with invasive cancer were marked on H&E-stained slides and two 0.6-mm tissue cores were taken from each donor block and mounted in triplicate recipient blocks using an automated arrayer (ATA-27, Beecher, Inc., Sun Prairie, WI).

Immunohistochemistry. Four-micrometer sections were dried, deparaffinized, rehydrated, and microwave treated in citrate buffer (pH 6.0) before processed in an automated staining machine (Techmate 500, DAKO, Copenhagen, Denmark) using the monoclonal antibody cyclin A2 (H432, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and cyclin D1 (Clone DSC-6, 1:100, DAKO A/S, Glostrup, Denmark). Evaluation of cyclin A2 was possible in 405 cases as the fraction of positively staining nuclei subdivided into five groups: 0 (0-1%), 1 (2-10%), 2 (11-25%), 3 (26-50%), and 4 (>50%). Cyclin D1 was evaluable in 463 cases and reported as the nuclear fraction and -intensity as well as cytoplasmic intensity. The nuclear fraction was subgrouped as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (>75%) and the nuclear and cytoplasmic intensity as absent = 0, weak = 1, intermediate = 2, and strong = 3. The TMAs had previously been analyzed immunohistochemically for ER and progesterone receptor (PR) status using the Ventana Benchmark system (Ventana Medical Systems, Inc., Tucson, AZ) with prediluted antibodies (anti-ER Clone 6F11 and anti-PgR Clone 16). In line with the clinically established cutoff used for hormone receptor assessment, tumors with >10 % positively stained nuclei were considered positive. Ki-67 index had been assessed previously using a monoclonal antibody (Ki-67; 1:200, M7240, DAKO, Glostrup, Denmark).

Fluorescence *in situ* hybridization. For FISH analysis of *CCND1* gene amplification, two direct-labeled probes were used, LSI cyclin D1 (11q13) SpectrumOrange against CCND1 (11q13) and CEP 11 SpectrumGreen (Vysis, Inc., Downers Grove, IL) against the centromere of chromosome 11. The FISH analysis was done according to "LSI Locus Specific Identifier DNA probes" from Vysis. Briefly, tissue sections were deparaffinized in xylene and alcohol and air-dried. The slides were then microwave treated in Target Retrieval Solution pH 7.3 (DAKO, Glostrup, Denmark) for 5 + 5 minutes and treated with 100 μL pepsin (Digest-All 3, Zymed, San Francisco, CA) for 8 minutes in 37°C before denaturing in 70% formamide/2× SSC at 73°C for 5 minutes. A mixture of 1 μL LSI probe, 2 μL pH_2O, and 7 μL LSI hybridization buffer was then added and incubated at 37°C overnight before washes.

A minimum of 50 nonoverlapping nuclei were scored and the *CCND1* gene was considered amplified when the ratio of orange/green signals was >1 in at least 20% of tumor cells. Nonamplified cases were classified as 0, cases with up to 10 copies as 1 and >10 copies as 2. In addition, 50 randomly selected tumors were analyzed using 1.0-mm cores, without increasing the number of valid cases and whole tissue sections from 50 additional tumors were analyzed to validate the array data. The signal intensity was all over weaker in whole sections, but the findings did not deviate from the arrayed specimens.

Statistics. Baseline prognostic and clinical characteristics between patients with and without available FISH data were compared using the χ^2 test to exclude selection bias. The same approach was used when comparing amplified and nonamplified cases and marker distribution according to trial arm. All survival analyzes were done with the intention to treat rule. Recurrence-free survival considered local, regional, distant recurrences and breast cancer specific death, but not contralateral breast cancer, as primary event. The Kaplan- Meier method was used to estimate recurrence-free survival and overall survival and the log-rank test to compare survival in different strata. A Cox proportional hazards model was used for the estimation of relative risks (RR) in univariate and multivariate analysis. The interaction between tamoxifen treatment and the investigated variables was further explored by a Cox model including one of the four variables, respectively, a treatment variable and an interaction variable. All statistical tests were two sided. Calculations were done with SPSS 11.0 (SPSS, Inc., Chicago, IL).

Results

Cyclin D1, cyclin A2, and clinicopathologic variables. Immunohistochemical evaluation of cyclin A2 was possible in 405 cases and of cyclin D1 in 463 cases. The *CCND1* gene status could be assessed in 280 cases (56% of the cases in the present study) and 44 cases (15%) were amplified, 10 of these (2.8%) with a copy number of >10. Apart from the 64 initially missing tumors, CCND1 status could not be determined in 220 tumors, despite analyses on repetitive sections. Examples of immunohistochemical staining of cyclins D1 and A2 as well as FISH analyses of CCND1 copy numbers are shown in Fig. 1*A-C*. There was no difference in marker distribution or clinicopathologic variables between the two trial arms (data not shown). Cyclin D1 protein was more frequently overexpressed in ER-positive tumors and cyclin A2 in ER-negative tumors. The nuclear fraction, intensity, and cytoplasmic intensity of cyclin D1 were strongly and significantly interrelated (data not shown) and cyclin D1 was negatively associated with proliferation except for in the subgroup of ER-positive tumors. Cyclin A2 was positively associated with Ki-67, histologic grade (NHG) and inversely associated with PR status, whereas cyclin D1 protein expression and amplification were positively associated with PR. *CCND1* gene amplification further correlated significantly with cyclin D1 protein expression and the majority of amplified tumors (42of 44, 95.5%) were ER positive. Importantly, there was no significant difference regarding baseline clinicopathologic characteristics in cases with and without CCND1 amplification data (data not shown). The relationship of clinicopathologic variables as well as immunohistochemical marker expression in relation to CCND1 amplification status is shown in Table 1.

Cyclin D1, cyclin A2, and tamoxifen response. In line with our previous findings in postmenopausal women (18), recurrence-free survival was not improved upon tamoxifen treatment in tumors with a strong nuclear cyclin D1 staining intensity, in contrast to a significant tamoxifen response in tumors with an absent to moderate staining intensity (Table 2; Fig. 2*B*). Cyclin D1 expression defined by the nuclear fraction did not influence the effect of tamoxifen treatment (Table 2), irrespective of the cutoff used. In contrast, tumors with <10% cyclin A2–positive nuclei responded well to tamoxifen, whereas tumors with >10% cyclin A2–positive nuclei had no significant response (Fig. 2; Table 2). However, the implied interference between tamoxifen treatment and cyclin D1 and cyclin A2 protein expression, respectively could not be established in a multivariate interaction analysis as detailed in Table 3.

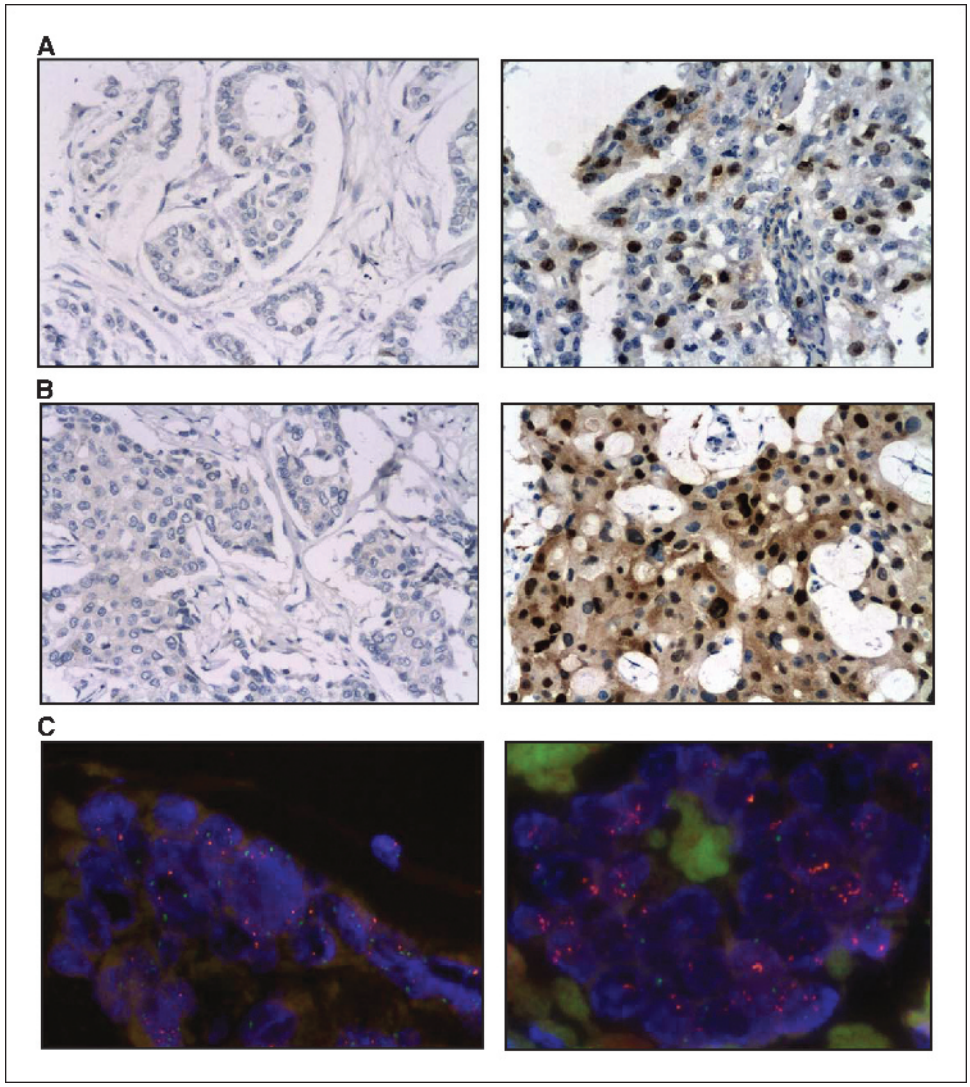

Figure 1. Examples of immunohistochemical staining and FISH analysis, with negative or unamplified cases (*left*) and positive or amplified cases (*right*). *A,* cyclin A2; *B,* cyclin D1; and *C, CCND1* gene copy numbers.

Surprisingly, we found that amplification status of the *CCND1* gene was the most powerful predictor of tamoxifen response in this study. As shown in Table 2 and Fig. 3, patients with amplified tumors tended to have an adverse outcome upon tamoxifen treatment in univariate analysis of recurrence-free survival (RR, 2.22; $P = 0.06$), in contrast to an excellent response associated with the absence of amplification (RR, 0.39; $P < 0.0001$). For patients with nonamplified tumors, the proportional 10-year recurrence-free survival was 74% in the treatment arm compared with 44% in the control arm. In contrast, 29% of the tamoxifen-treated patients with CCND1-amplified tumors were recurrence free at 10 years compared with 62% in the untreated group. In multivariate interaction analysis, a significant interaction between tamoxifen treatment and CCND1 amplification was observed both for recurrence-free survival and overall survival (Table 3).

Because the number of node positive patients was rather high in this study (402 of 564, 71%), we also explored this high-risk group separately and found a significantly shorter recurrence-free survival as well as overall survival in tamoxifen-treated patients with CCND1-amplified tumors, whereas patients with nonamplified tumors still had an excellent outcome after tamoxifen treatment (Fig. 3). For node-positive patients, the 10-year recurrence-free survival was 68% in the untreated compared with 13% in the treated arm. Consequently, the multivariate interaction variable was even more significant for node-positive patients with a >9-fold difference in treatment response for CCND1-amplified tumors.

Despite the strong association between *cyclin D1* gene amplification and protein content, defined by the nuclear intensity or nuclear fraction, the treatment-predictive values of these variables differed considerably, with a suggested adverse tamoxifen effect in amplified tumors, no effect in tumors with a strong nuclear intensity, and a beneficial effect in tumors with a high nuclear fraction of cyclin D1. In an attempt to address this issue, we further tried to define subgroups of cyclin D1 alterations and interestingly, tamoxifen treatment in CCND1-amplified tumors with a low protein content, defined either as a low/moderate nuclear intensity ($n = 30$) or <50% positive nuclei ($n = 24$), was associated with a significantly shorter recurrence-free survival ($P = 0.025$ and 0.026, respectively). No conclusions could be made regarding the few tumors with a high cyclin D1 nuclear intensity but no *CCND1* gene amplification, due to very few events in this subgroup. Nevertheless, for tumors without CCND1 amplification but with a high cyclin D1 protein content ($n = 30$), defined either as a strong nuclear intensity or >50% positive cells, there was a

Table 1. Clinicopathologic characteristics and marker distribution according to CCND1 amplification status for all and ER-positive tumors

	CCND1 amplified, n = 44 (%)	CCND1 nonamplified, n = 236 (%)	P	CCND1 amplified ER$^+$, n = 42 (%)	CCND1 nonamplified ER$^+$, n = 151 (%)	P
Age (y)						
Median (range)	44.5 (33-52)	44 (25-57)	0.81*	44.5 (33-52)	45 (26-57)	0.78*
<40	7 (15.9)	52 (22.0)	0.53†	6 (14.3)	27 (17.9)	0.59†
40-49	32 (72.7)	151 (64.0)		31 (73.8)	99 (65.6)	
>50	5 (11.4)	33 (13.0)		5 (11.9)	25 (16.5)	
Randomization						
Tamoxifen	23 (52.3)	111 (47.0)	0.63†	21	72 (47.7)	0.79†
Control	21 (47.7)	125 (53.0)		21	79 (52.3)	
Tumor size (mm)						
Median (range)	20.5 (4-50)	25 (2-55)	0.11*	20 (4-50)	23 (7-50)	0.32*
<20	22 (50.0)	91 (38.6)	0.21†	22 (5.5)	64 (42.4)	0.25†
>20	22 (50.0)	145 (61.4)		20 (47.6)	87 (57.6)	
Node status						
0	8 (18.2)	67 (28.4)	0.02†	8 (19.0)	32 (21.2)	0.42†
1-3	28 (63.6)	114 (48.3)		27 (64.3)	81 (53.6)	
>4	7 (15.9)	55 (23.3)		7 (16.7)	38 (25.2)	
Not evaluated	1 (2.3)	2		—	—	
NHG						
1	2 (4.5)	34 (14.4)	0.001†	2 (4.8)	30 (19.9)	0.03†
2	30 (68.2)	86 (36.4)		29 (69.0)	75 (49.7)	
3	12 (27.3)	109 (46.2)		11 (26.2)	45 (29.8)	
Not evaluated	—	7 (3.0)		—	1 (0.6)	
ER						
Negative	1 (2.3)	80 (33.9)	<0.001	—	—	—
Positive	42 (95.4)	151 (64.0)		—	—	
Not evaluated	1 (2.3)	5 (2.1)		—	—	
PR						
Negative	5 (11.4)	82 (34.7)	0.001	3 (7.1)	14 (9.3)	0.89†
Positive	38 (86.4)	141 (59.7)		38 (90.5)	132 (87.4)	
Not evaluated	1 (2.3)	13 (5.5)		1 (2.4)	5 (3.3)	
Ki-67 index (%)						
0-1	2 (4.5)	36 (15.2)	0.03†	2 (4.8)	24 (15.9)	0.27†
2-10	21 (47.8)	68 (28.8)		20 (47.6)	57 (37.8)	
10-25	15 (34.1)	53 (22.4)		15 (35.7)	35 (23.2)	
25-50	4 (9.1)	26 (11.0)		4 (9.5)	16 (10.6)	
>50	2 (4.5)	30 (12.7)		1 (2.4)	4 (2.6)	
Not evaluated	—	23 (9.7)		—	15 (9.9)	
D1 nuclear fraction (%)						
0	0	56 (23.7)	<0.001†	0	10 (6.6)	<0.001†
1-25	9 (20.4)	100 (42.4)		9 (21.4)	71 (47.0)	
26-50	15 (34.1)	49 (20.8)		14 (33.13)	43 (28.5)	
51-75	11 (25.0)	23 (9.7)		11 (26.2)	21 (13.9)	
>75	8 (18.2)	4 (1.7)		8 (19.0)	4 (2.6)	
Not evaluated	1 (2.3)	4 (1.7)		—	2 (1.3)	
D1 nuclear intensity						
0	0	56 (23.7)	<0.001†	0	10 (6.6)	<0.001†
1	8 (18.2)	91 (38.6)		8 (19.0)	61 (40.4)	
2	22 (50.0)	70 (29.7)		21 (50.0)	64 (42.4)	
3	13 (29.5)	15 (6.3)		13 (31.0)	14 (9.3)	
Not evaluated	1 (2.3)	4 (1.7)		—	2 (1.3)	
D1 cytoplasmic intensity						
0	1 (2.3)	16 (6.8)	0.04†	0	2 (1.3)	0.31†
1	13 (29.5)	96 (40.7)		13 (31.0)	50 (33.1)	
2	14 (31.8)	83 (35.2)		14 (33.3)	65 (43.1)	
3	15 (34.1)	37 (15.7)		15 (35.7)	32 (21.2)	
3+++	0	2 (0.8)		0	2 (1.3)	
Not evaluated	1 (2.3)	2 (0.8)		—	—	

(*Continued on the following page*)

Table 1. Clinicopathologic characteristics and marker distribution according to CCND1 amplification status for all and ER-positive tumors (Cont'd)

	CCND1 amplified, *n* = 44 (%)	CCND1 nonamplified, *n* = 236 (%)	*P*	CCND1 amplified ER$^+$, *n* = 42 (%)	CCND1 nonamplified ER$^+$, *n* = 151 (%)	*P*
A2 nuclear fraction (%)						
0-1	1 (2.3)	30 (12.7)	0.04 †	1 (2.4)	21 (13.9)	0.14 †
2-10	23 (52.3)	78 (33.0)		22 (52.4)	61 (40.4)	
11-25	16 (36.3)	75 (31.8)		16 (38.1)	46 (30.5)	
26-50	1 (2.3)	24 (10.2)		0	5 (3.3)	
51-100	0	2 (0.8)		0	1 (0.7)	
Not evaluated	3 (6.8)	27 (11.4)		3 (7.1)	17 (11.2)	

*Mann-Whitney *U* test for comparison of medians.
† χ^2 test.

significantly positive tamoxifen effect regarding recurrence-free survival ($P = 0.048$), further supporting that CCND1 amplification rather than a high protein expression may be indicative of an agonist function of tamoxifen.

Prognostic information of cyclin D1 and cyclin A2. A high cyclin A2 expression was inversely associated with survival in the untreated group, both recurrence-free survival (RR, 1.34; 95% CI, 0.91-1.96; $P = 0.13$) and overall survival (RR, 1.66; 95% CI, 1.11-2.48; $P = 0.01$), which is line with findings from earlier studies (17, 24) and with proliferation markers in general. In contrast, cyclin D1 protein content or CCND1 amplification did not confer any significant prognostic information, neither in the subset of ER-positive tumors, although there was a trend towards a more favorable outcome for tumors with CCND1 amplification (data not shown).

Discussion

By high-throughput tissue analyses of tumors from premenopausal women included in a randomized adjuvant tamoxifen trial with long-term follow-up, we have found that *CCND1* gene amplification, a nonrandom genetic alteration occurring in about

Table 2. Recurrence-free and overall survival in ER-positive patients by Cox univariate analysis

Category	*n*	Recurrence-free survival		Overall survival	
		RR (95% CI)	*P*	RR (95% CI)	*P*
Cyclin D1 nf <25%					
Control	83	1.00		1.00	
Tamoxifen	56	0.62 (0.37-1.04)	0.07	0.65 (0.37-1.14)	0.14
Cyclin D1 nf >25%					
Control	84	1.00		1.00	
Tamoxifen	88	0.61 (0.38-0.96)	0.03	0.78 (0.48-1.25)	0.30
Cyclin D1 ni low/intermediate					
Control	149	1.00		1.00	
Tamoxifen	120	0.59 (0.41-0.85)	0.004	0.72 (0.50-1.06)	0.10
Cyclin D1 ni high					
Control	18	1.00		1.00	
Tamoxifen	24	0.84 (0.30-2.31)	0.73	0.77 (0.27-2.19)	0.62
Cyclin A2 nf <10%					
Control	90	1.00		1.00	
Tamoxifen	69	0.51 (0.30-0.86)	0.01	0.72 (0.42-1.25)	0.25
Cyclin A2 nf >10%					
Control	52	1.00		1.00	
Tamoxifen	51	0.86 (0.50-1.47)	0.59	0.92 (0.53-1.59)	0.75
CCND1 not amplified					
Control	79	1.00		1.00	
Tamoxifen	72	0.39 (0.23-0.65)	<0.0001	0.43 (0.24-0.76)	0.004
CCND1 amplified					
Control	21	1.00		1.00	
Tamoxifen	21	2.22 (0.94-5.26)	0.06	2.13 (0.89-5.10)	0.09

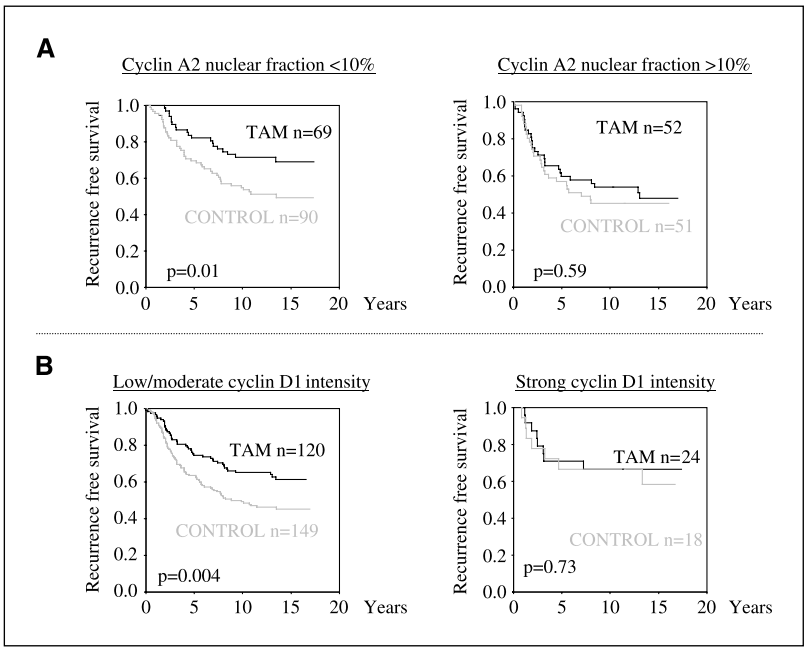

Figure 2. Recurrence-free and overall survival for ER-positive cases with and without tamoxifen treatment according to cyclin A2 expression (*A*) and cyclin D1 expression (*B*).

15% of all breast cancer, may predict an agonistic rather than antagonistic effect of tamoxifen treatment. The findings were further strengthened in multivariate interaction analyses, showing a 6- to 9-fold higher relative risk of disease recurrence and death in CCND1-amplified tumors, which is statistically highly significant. This discrepancy was even more accentuated in node-positive patients, constituting a relatively large number (71%) of the patients in this study. Furthermore, this remarkable adverse effect was obtained after only 2 years of adjuvant tamoxifen treatment.

In this study, amplification data could not be retrieved in 44% of the analyzed tumors, even in consecutive sections or manually constructed TMAs with a larger core diameter (1.0 mm). Whole tissue sections were analyzed in 50 randomly selected cases to validate the TMA data, but this approach was not applicable on specimens with uncertain or absent signals in the TMAs, because signals were generally weaker in whole tissue sections. This suggests that a fraction of the tumors simply could not be analyzed by FISH technology, probably due to a variability in tissue fixation or other steps in the tissue processing, which was not standardized at the time of the primary handling of these tumors (1984-1991). Nevertheless, a success rate of 56% for FISH analysis on arrayed archival tissue is compatible with other studies, reporting success rates from 34% to 82% for FISH analyses of various genes on different tumor types in TMAs (25–27). As discussed in these and other studies and according to our own experience, the most common reason for noninterpretable results is weak hybridization, which is not related to the TMA technology but also seen in whole sections. The TMA technique itself is associated with some additional technical problems, such as sample detachment and a great variance in the pretreatment needed by each sample.

Table 3. A multivariate Cox proportional hazards model for cyclin A2, cyclin D1, CCND1 status and treatment interaction

Variable		Recurrence free survival		Overall survival	
		RR (95% CI)	*P*	RR (95% CI)	*P*
D1 NF	Low versus high	0.94 (0.62-1.44)	0.79	0.85 (0.54-1.34)	0.49
Treatment	Tamoxifen versus control	0.67 (0.39-1.12)	0.13	0.67 (0.38-1.19)	0.17
Interaction variable	Tamoxifen × D1 nf	0.88 (0.44-1.79)	0.74	1.12 (0.53-2.37)	0.76
D1 NI	Low/moderate versus high	0.80 (0.36-1.74)	0.57	0.96 (0.44-2-20)	0.92
Treatment	Tamoxifen versus control	0.62 (0.42-0.88)	0.009	0.73 (0.49-1.08)	0.11
Interaction variable	Tamoxifen × D1 ni	1.18 (0.40-3.50)	0.77	0.84 (0.27-2.58)	0.76
A2 NF	Low versus high	0.97 (0.58-1.62)	0.92	1.12 (0.65-1.93)	0.68
Treatment	Tamoxifen versus control	0.48 (0.28-0.82)	0.007	0.68 (0.39-1.19)	0.18
Interaction variable	Tamoxifen × A2 nf	1.78 (0.83-3.79)	0.14	1.25 (.57-2.75)	0.58
CCND1	Amplified versus nonamplified	0.57 (0.27-1.22)	0.15	0.82 (0.30-1.79)	0.62
Treatment	Tamoxifen versus control	0.39 (0.23-0.65)	<0.001	0.41 (0.22-0.75)	0.004
Interaction variable	Tamoxifen × CCND1	6.38 (2.29-17.78)	<0.001	5.34 (1.84-15.51)	0.002

NOTE: Adjusted for age (continuous), tumor size (continuous), NHG (1 + 2 versus 3), and nodal status (0 versus 1).

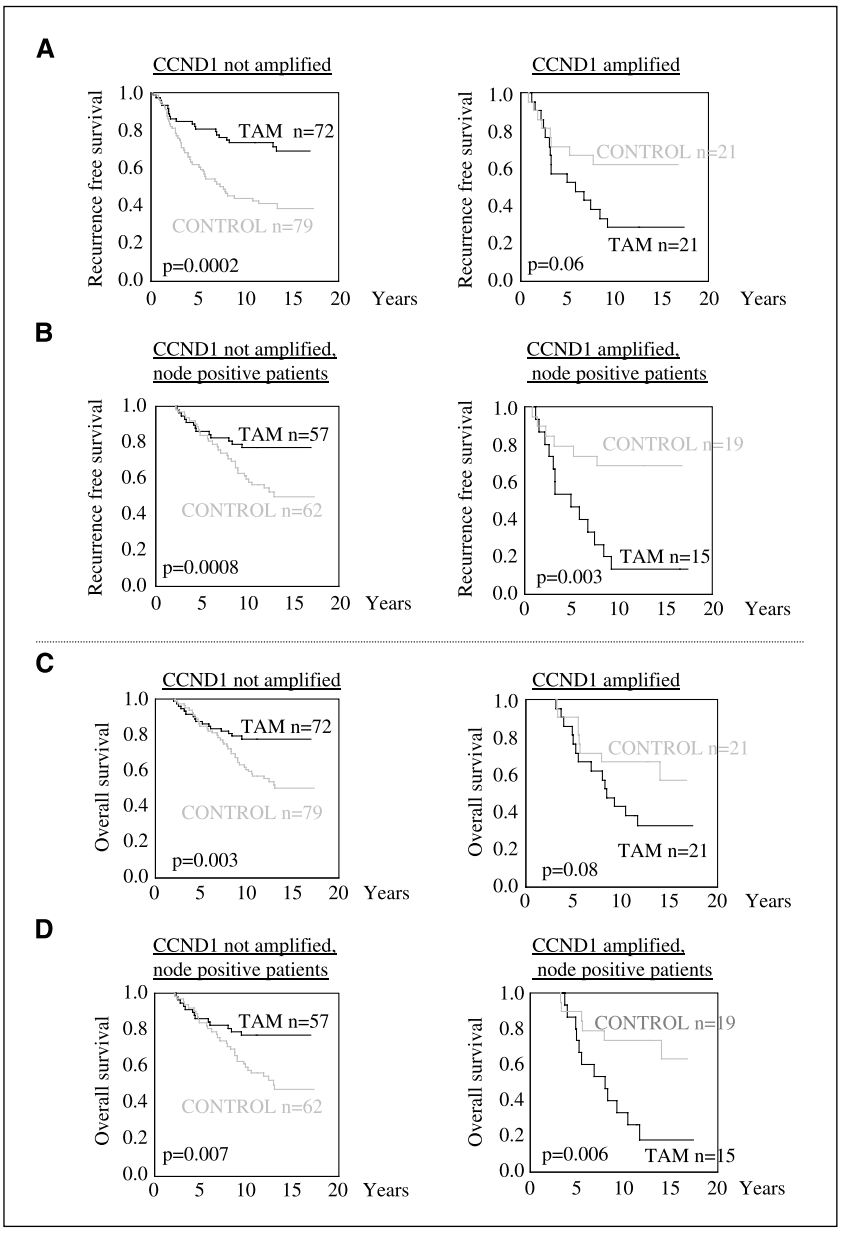

Figure 3. Recurrence-free (*A* and *B*) and overall survival (*C* and *D*) for ER-positive cases with and without treatment according to *CCND1* gene copy numbers (*A* and *C,* all patients; *B* and *D,* lymph node–positive cases).

Although such a loss of information can never be fully compensated for, we have been able to show that the noninformative cases in this study did not differ from the successfully analyzed group regarding important tumor characteristics thus substantially reducing the likelihood of a selection bias (data not shown).

The *CCND1* gene is located at chromosome 11q13, a gene-dense region that seems amplified in a variety of human malignancies (28, 29). Several large studies on breast tumors have established at least four major cores of amplification within the *11q13* locus (30–32) and in general, 11q13 amplifications may involve amplification of a large region spanning several cores. The CCND1 region is the most frequently amplified, constituting about two thirds of all 11q13 amplifications. In this study, the frequency of CCND1 amplification was 15%, which is in concordance with previously reported rates (33–35). In breast cancer, the two most eligible key oncogenes on this amplicon are cyclin D1 and EMS1 (6, 28, 30, 36, 37), the latter encoding the human homologue of the cytoskeletal actin-binding protein and *c-Src* substrate Cortactin (38). CCND1 and EMS1 amplification seem to confer different phenotypes in ER-positive and -negative breast cancer and EMS1 amplification has been associated with early relapse in lymph node–negative and ER-negative disease (39). Considering this complexity of *11q13* amplification patterns, a more comprehensive mapping of the functional genes within this locus is needed to elucidate whether CCND1 amplification is the primary event associated with an agonist effect of tamoxifen, as implied in this study, or if it merely reflects the coamplification of another, more crucial, gene and corresponding overexpression of a protein not yet identified. Other approaches to define genes that are involved in tamoxifen resistance in breast cancer have defined certain gene expression clusters that could be relevant in predicting tamoxifen response (40, 41). However, these candidate genes do not match any of the proteins investigated in this study and are not linked to the 11q13 amplicon.

Although cyclin D1 protein expression correlated strongly with *CCND1* gene amplification in this study, the latter was by far the most powerful predictor of tamoxifen response, even in the absence of protein overexpression. These findings indicate that the cyclin D1 protein may not primarily be involved in the altered tamoxifen response, despite its strong link to the ER and experimental data suggesting a direct interaction between cyclin D1 and ER. Nevertheless, until more is known about the role of the 11q13 amplicon in endocrine resistance, amplification status of the CCND1 gene seems an eligible marker for identifying tumors in which tamoxifen may have an agonistic effect. Furthermore, FISH analysis of the CCND1 gene copy number on formalin fixed tissue is a fairly standardized technology that could easily be implemented into routine pathology protocols at centers handling FISH analyses of the *HER2* gene today.

However, further retrospective and prospective studies, also involving postmenopausal breast cancer patients, are needed to validate and potentially confirm our data. This study also shows that tamoxifen is an extremely efficient adjuvant treatment for non-CCND1-amplified ER-positive tumors and definitely has a role also in future breast cancer treatment regimens.

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