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The prognostic value and overexpression of cyclin A is correlated with gene amplification of both cyclin A and cyclin E in breast cancer patient

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Abstract. Deregulation of cell cycle control is a hallmark of cancer. The primary cyclins (A, B1, D1, D3 and E) are crucial for cell cycle progression. Secondary cyclins (C and H) have putative indirect effects on cell cycle propulsion and are not previously evaluated in breast cancer. We have examined protein expression and gene amplification of cyclins in breast carcinomas and correlated the findings with clinical follow-up data. We have previously demonstrated that over-expression of cyclin A is associated with poor prognosis in breast cancer patients. In this study we wanted to evaluate the mechanisms behinde overexpression of cyclin A, as well as the impact of other cyclins, both at the gene level and at the protein level, on prognosis of breast cancer patients. The impact of TP53 gene mutations on gene amplification of cyclins was also evaluated. *Methods*: Real-Time Quantitative PCR was used to detect gene amplification of cyclins in tumour tissue from 86 patients operated for invasive breast carcinomas, while immunohistochemistry was applied to detect protein expression of the same cyclins. *Result*: Of the 80-breast tumour samples available for cyclin A gene amplification analyses, 26.7% (23/80) was defined to have cyclin A gene amplification. 37.2% (32/79) had cyclin B1 gene amplification, 82.6% (71/82) of the samples harboured amplification of cyclin C gene, 74.4% (64/82) had cyclin D1 gene amplification, 41.9% (36/86) had cyclin D3 gene amplification, 29.1% (25/81) of the patients had cyclin E gene amplification and 9.3% (8/86) of the samples showed amplification of the cyclin H gene. When correlation between gene amplification and protein expression was evaluated, we observed a statistical significant correlation between gene amplification and protein expression of cyclin A ($p = 0.009$) and cyclin D3 ($p < 0.001$). However, the correlation between gene amplification and protein expression of cyclin A, as well as the prognostic value of cyclin A overexpression, was affected by gene amplification of cyclin E. Gene amplification of none of the other cyclins was associated with patient prognosis. There was a statistical significant correlation between TP53 gene mutations and gene amplification of cyclins A, D3 and B1. No correlation was observed between gene amplification of secondary cyclins (H and C) and TP53 gene mutations. *Conclusions*: The overexpression of cyclin A is correlated to gene amplification of both cyclin A and cyclin E. Over-expression of cyclin A is associated with poor prognosis in breast cancer patients. When analysed in a multivariate analyses model, gene amplification as well as protein expression of none of the other cyclins than cyclin A are associated with patient prognosis in breast carcinomas. TP53 gene mutation seems to correlate with gene amplification of primary, but not secondary cyclins.

Keywords: Cyclins, gene amplification, immunohistochemistry, patient survival

1. Introduction

Cell proliferation is fundamentally the result of repeated progression through a scheduled series of events, named cell cycle. Cell cycle is regulated by the action of defined protein complexes. Deregulation of the cell-cycle control is a hallmark of cancer [16]. The control of the cell-cycle depends upon cyclin dependent kinases (Cdks), which function after complex-formation with cyclins. Cell cycle is theoretically defined as consisting of four phases; G0/G1 phase, S-phase, G2-phase, and M-phase. The G1-phase

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of the cell cycle is regulated by cyclin D- and E-associated kinases. Cyclin A-Cdk2 complexes take over at the G1/S-transition, allowing S-phase entry and replication. Mitosis is triggered by Cdk1, which is activated by a multistep process beginning with the binding of cyclin B1 [31].

The primary cyclins (A, B1, D1, D3 and E) have been studied in various malignancies, including breast cancer [1,7,13,14,17,24,30,38,41] but only one or a few cyclins have been included in each study. Despite the accumulated observations, reports describing the involvement of these molecules in oncogenesis and tumour cell proliferation in human carcinomas have not been fully consistent. Although up-regulation of Cdc2 and cyclin A have been shown to indicate a negative prognostic outlook [2,10,34,41], the relationship between overexpression of other cyclins and clinicopathological outcome varies considerably, depending on the tumour type and patient subsets. For example, cyclin E was once designed as a negative prognostic marker in carcinoma of the colon, ovary, and breast [22,23,29,32,35] their studies reported that its up-regulation is a positive prognostic factor [38].

Cyclin C has a putative phase G1 cyclin function [26,27]. However, cyclin C can also regulate both cell cycle progression as well as gene transcription: Cyclin C form complexes with Cdk8 and induces transcription of Cdc2 (Cdk1). Cdk8/cyclin C is a component of RNA polymerase II holoenzyme complex where they function as a kinase that phosphorylates the carboxyterminal domain [19] and can also repress transcription by phosphorylating the Cdk7/cyclin H subunits of the general transcription initiator factor IIH (TFIIH) [3]. The Cdk7/cyclin H can activate many other cyclin/Cdk complexes immediately involved in the cell cycle progression as Cdk2, Cdk4 or Cdc2 [15]. There had been a limited number of reports concerning the secondary cyclins C and H in clinical materials. The Cdk8 gene has been linked to lymphoblastic leukemias [28] and the cyclin C protein has been associated with the pathogenesis of Alzheimer's disease [39]. The prognostic value of cyclin C and cyclin H over-expression, as well as gene amplification has not been evaluated previously.

We have previously demonstrated that overexpression of cyclin A is a marker of poor prognosis in breast cancer patients [7]. The overexpression of cyclin A overrides the prognostic effect of impaired p53 [8] function and of β-catenin [6]. In this study we wanted to evaluate the mechanisms behind the over-expression of the protein. Cyclin A, as a member of genes involved in cell cycle control, requires coordinated activation and repression of specific sets of transcription factors, among which members of the E2F/DP family constitute a thoroughly studied archetype [12]. Cell cycle modulation of the cyclin A expression is due, in part, to the periodic relief of a transcriptional repression when cells enter S phase. Cyclin A belongs to groups of genes whose transcription is repressed in G0/early G1 and de-repressed either in late G1, during S phase, or as cells transit into G2. There are evidences for that in addition to through E2F/DP system regulation, expression of cyclin A may also be modulated by expression of cyclin E through the modulation of pRB inhibition of S phase entry [4].

In the present study, we have mapped the protein expression and gene amplification status of the cyclins exhibiting a potential role in regulation of the cell cycle (cyclins A, B1, C, D1, D3, E and H) in breast carcinomas and their prognostic value in a cohort of breast cancer patients with more than 10 years of follow up.

2. Patients

The patient cohort included in this study is part of a cohort previously described [7]. 86 of the patients operated at the Akershus University Hospital in the period 1988–1990 were included. Mean age at the diagnosis was 55.4. Eight of the tumours were classified as lobular invasive, 62 as ductal invasive, and 10 as other types. Lymph node dissection was performed in all of the patients, of which 50 were lymph node negative and 36 were lymph node positive. 9 were classified as histology grade I, 50 as histology grade II and 27 as histology grade III. 34 of the tumours were oestrogen receptors negative, and 52 oestrogen receptors positive.

The survival follow up time was more than 14 years. 43 patients died during the follow up time. Of these 27 died of breast cancer.

2.1. Immunohistochemistry

The immunohistochemistry methods are described previously [7] as well as outlined in Table 1. Briefly, four to six μm thick sections from formalin-fixed, paraffin-embedded tumour tissue obtained at the time of surgery were made on coated slides. After antigen retrieval by microwave technique (2×5 minutes), the immunostaining was performed in an Optimax plus, Automated Cell Stainer; Model 1.5 (BioGenix, USA) following the operating manual. All series included

Table 1
Antibodies and working conditions

Antibody	Dilution	Sources	Pretreatment
Cyclin A	1 : 50	Novocastra, UK	2 × 5 min microwave, 1 mM EDTA (pH 8)
Cyclin B1	1 : 200	BioSource International, USA	5 + 15 min 350 W microwave, 10 mM citrate buffer (pH 6)
Cyclin C	1 : 50	Transduction Laboratories, USA	5 + 15 min 350 W microwave, 10 mM citrate buffer (pH 6)
Cyclin D1	1 : 200	Oncogene Research, NY	2 × 5 min microwave, 1 mM EDTA buffer (pH 8)
Cyclin D3	1 : 25	Dako, CA	4 × 5 min microwave, 1 mM EDTA (pH 8)
Cyclin E	1 : 100	Santa Cruz Biotechnology, CA	4 × 5 min microwave, 2 mM citrate buffer (pH 6)
Cyclin H	1 : 300	Santa Cruz Biotechnology, CA	5 + 15 min 350 W microwave, Tris/EDTA (pH 9)

positive and negative controls. Only cells with staining of the nuclei were scored as positive. The number of immunoreactive cells was estimated semiquantitatively. Grade + corresponded to 5–30% positive cells, grade ++ to 30–70% positive cells and grade + + + to >70% positive cells.

2.2. TP53 gene mutations

Direct sequencing was performed to detect mutations in exon 5–8 of the TP53 gene. The method and results are previously described [8]. DNA from paraffin embedded tissue sections was extracted using the GenoM™-48 Robotic Workstation according to the method described by the manufacturer (GenoM™-48, Automated DNA Isolation from Tissue Handbook, February 2001, GenoVision, Oslo, Norway). After genomic DNA was prepared from the samples, the DNA was quantitated by ultraviolet spectroscopy in a GeneQuantpro™ spectrophotometer (Amersham Pharmacia Biotech, Buckinghamshire, GB).

2.3. Real-Time PCR

The quantification of DNA was carried out using a real-time fluorescence detection method [18]. Real-Time quantitative PCR analyses were preformed using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The Polymerase chain reaction (PCR) primers and TaqMan fluorogenic probes for ccnA2, ccnB1, ccnC, ccnD1, ccnD3, ccnE1 and ccnH were chosen with the assistance of the Primer Express 2.0 software (Applied Biosystems). For each primer and probe, we conducted a BLASTN search against the GenBank database to confirm the total gene specificity and the absence of DNA polymorphisms. Primers were purchased from Invitrogen (Carlsbad, CA, USA) and probes from Applied Biosystems. Primer and probe combinations were positioned to span an exon–intron junction. Their sequences are shown in Table 2. The Human Serum Albumin gene (HSA) was employed as an endogenous reference gene. No genetic alterations have been found by means of CGH in the chromosome region 4q11–13, where the HSA gene is encoded. The HSA primers and probe are previously described. DNA from normal breast tissue was used as a calibrator sample. PCR was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems) using 1 μl DNA (2–50 ng/μl), 200 nm probe, 600 nM primers (except for HSA sense primer and HSA antisense primer which was used at 700 nM) in a 25 μl final reaction volume. Experiments were preformed in triplicates for each sample. The calibrator sample was analysed on every assay plate together with the patient samples and a negative control. Default thermal cycling conditions were used in the PCR (Applied Biosystems).

2.4. Comparative CT method and determination of relative gene copy number

The $2^{-\Delta\Delta\mathrm{Ct}}$ method was employed to analyse the relative changes in gene copy number using real time quantitative PCR. According to this method the relative gene copy number is calculated by the following equation: $2^{-(\Delta\Delta\mathrm{Ct})}$, were $\Delta\Delta\mathrm{Ct} = (\Delta\mathrm{Ct}$ albumin (calibrator sample) $- \Delta\mathrm{Ct}$ cyclin (calibrator sample)) $-$ (Ct albumin (patient sample) $- \Delta\mathrm{Ct}$ cyclin (patient sample) (Applied Biosystems User Bulletin No. 2 (P/N 4303859). The variation in Ct value was consistently less than one, therefore the cut-off value for amplification was set at 2. The data provided from this formula thus represents the fold-change in gene number normalized to the endogenous reference (HSA) gene and relative to the calibrator sample (normal breast tissue). A validation experiment (Applied Biosystems User Bulletin No. 2 (P/N 4303859)) was performed confirming approximately equal efficiencies of target and reference gene amplification, implying that the $2^{-\Delta\Delta Ct}$ method is suitable.

Table 2
Primer and probe sequences used in the real time PCR of the indicated genes

Gene		Primer sequence (5′–3′)	Hybridisation probe sequence (5–3′)
Cyclin A2	sense	AAGAAGCCAGCTGAATCTCAAA	AAAAGCCAGGGCATCTTCACGCTCTATT
	antisense	GGTCCAGGTAAACTAATGGCTGAA	
Cyclin B1	sense	CCCTGCTGCAACCTCCAA	CCCGGACTGAGGCCAAGAACAGC
	antisense	TGTTCACTGACTTTGTTACCAATGTC	
Cyclin C	sense	GCCGGCTGGTGCTTTTTTA	TGCCATGGAACACAGCTTGCCCT
	antisense	TGGGAGCTCTGCCAAAAGTT	
Cyclin D1	sense	CCGTCCATGCGGAAGATC	CCTCCAGCATCCAGGTGGCGA
	antisense	AACAAGTTGCAGGGAAGTCTTAAGA	
Cyclin D3	sense	CTGTCTCTCCCCGCCAGTT	CACCCCCGACACGTATTGTCTCCC
	antisense	CTGATATCTCAAGCTTTCCTTTTCCT	
Cyclin E1	sense	CCCCGCTGCCTGTACTGA	TCAGTGCCGACTCTGCCACATGG
	antisense	AGCATGGAGTAAGAGACCTGGAA	
Cyclin H	sense	TATCCTTACCACACTTTTTTTCCTTCT	TCTACCAGGTCGTCATCAGTCCATTCTTCCT
	antisense	AGAAATCAACTTCAAATGGTTAGAGAGA	
HSA	sense	TGTTGCATGAGAAAACGCCA	AAGTGACAGAGTCACCAAATGCTGCACAG
	antisense	GTCGCCTGTTCACCAAGGAT	

All probes were labelled at the 5′ end with FAM as reporter and at the 3′ end with TAMRA as quencher.

2.5. Statistical methods

The cut off value for gene amplification was set to be 2.0 for statistical analyses. Samples with computed copy number levels above 2.0 were considered to reflect presence of gene amplification, whereas values below 2.0 were defined to represent non-amplified samples. Correlation analysis and logistic regression analysis was used to test the association between gene amplification and protein expression. For univariate analyses of survival the Kaplan–Meier model was used. For multivariate analysis of survival we used the Cox regression hazard model. Test for proportional hazard was performed to satisfy the criteria for use of the Cox model. In both the logistic regression model and Cox regression hazard model all genes were initially included in the analysis and one gene was step-wise omitted until only significant associations remained. Tests for interaction were included in the logistic regression model and the Cox regression hazard model. The statistical significant level was set at the p value 0.05. All statistical computations were performed with SPSS for Windows version 11.

3. Results

3.1. Gene amplification

Of the breast tumour samples available for gene amplification analyses, 26.7% (23/80) had cyclin A gene amplification. Of these 13 showed more than 2 copy numbers of the gene (2–6 copy numbers). 37.2% (32/79) cyclin B1 gene amplification, 29 of these were defined to have more than 2 copy numbers of the gene (2–7 copy numbers). 82.6% (71/82) cyclin C gene amplification, 78 of the tumours showed more than 2 copy numbers of the gene (2–9 copy numbers). 74.4% (64/82) cyclin D1 gene amplification, 61 of these were defined to have more than 2 copy numbers of the gene (2–13). 41.9% (36/86) cyclin D3 gene amplification, only 9 tumours showed more than 2 copy numbers of the gene (2–4). 29.1% (25/81) cyclin E gene amplification, 23 of the tumours showing more than 2 copy numbers of the gene (2–7). 9.3% (8/86) of the samples showed amplification of the cyclin H gene, 5 of these tumours showed more than 2 copy numbers of the gene (2–5).

3.2. TP53 gene mutations

TP53 gene mutation status has been described previously [28]. In this cohort, mutations in the TP53 gene were detected in 14 out of 86 tumour samples.

4. Immunohistochemistry

Immunohistochemistry results are part of a study previously described [7]. The immunostaining protocols are described in Table 1. In the present study,

76.7% of the tumour samples showed immunoreactivity to cyclin A (36+, 18++, and 11+++). 65.3% were positive to cyclin B1 antibodies (44+, 3++), 86.6% were immunoreactivity positive to cyclin C antibodies (11+, 24++ and 28+++), and for cyclin D1, immunoreactivity was detected in 13.4% of the tumour samples (1+, 9++, and 1+++). Immunoreactivity to cyclin D3 was detected in 41.9%, and for cyclin E in 48.1%, while immunoreactivity to cyclin H was observed in 98.6% (Table 3).

4.1. Correlation between immunohistochemistry and gene amplification

When correlation between gene amplification and protein expression of different cyclins was evaluated, we observed correlation between gene amplification and protein expression of cyclin A ($p = 0.009$) and of cyclin D3 ($p < 0.0001$). No correlation was observed between gene amplification and protein expression of cyclin D1 ($p = 0.98$), cyclin E ($p = 0.41$), and cyclin H ($p = 0.93$), while a borderline correlation was observed between gene amplification and protein expression of cyclin B1 ($p = 0.078$) and cyclin C ($p = 0.063$) (Table 3).

When correlation between TP53 gene mutations and gene amplification of different cyclins was evaluated, we observed a statistical significant correlation between TP53 gene mutations and gene amplification of cyclin A ($p = 0.002$), cyclin D3 ($p = 0.023$), cyclin B1 ($p = 0.049$), and borderline to cyclin D1 ($p = 0.075$). No correlation was observed between TP53 gene mutations and gene amplification of secondary cyclins (H and C), while gene amplification of cyclin A was correlated to gene amplification of cyclin C ($p = 0.025$) as well to gene amplification of cyclin D1 ($p = 0.002$) and cyclin E ($p = 0.0002$).

4.2. Prognostic value of gene amplification and protein expression

When the prognostic value of gene amplification of different cyclins was analysed, we observed that gene amplification of cyclin A ($p = 0.02$, Kaplan–Meier, univariate, HR 2.4; 95% CI: 1.077–5.324; $p = 0.032$ multivariate (Cox regression) adjusted for patient age, tumour grade, tumour type and lymph node metastases) was the only one associated with reduced patient survival. Gene amplification of none of the other cyclins had impact on patient survival. However, since there was a highly significant correlation between gene amplification of different cyclins, we performed a multivariate survival analysis, adjusted for gene amplification of the different cyclins. In this analysis, gene amplification of cyclin D1 showed an association to better prognosis when adjusted for cyclin A gene amplification, age, histological grade and tumour type (HR = 0.342, $p = 0.052$, 95% CI: 0.1–1.0), while unadjusted for cyclin A gene amplification, gene amplification of cyclin D1 failed to have any impact on the patient survival.

The impact of protein overexpression of different cyclins on cancer specific death in the present cohort of patients was also evaluated. Only cyclin A ($p = 0.003$ univariate (Kaplan–Meier), HR 2.3; 95% CI: 1.4–3–6; $p = 0.0004$ multivariate Cox regression) was associated with reduced patient survival, while over-expression of cyclin B1 ($p = 0.48$), cyclin C ($p = 0.49$), cyclin D1 ($p = 0.55$), cyclin E (0.22) and cyclin H ($p = 0.99$) failed to have any impact on patient survival.

4.3. Interactions between cyclin gene amplification, related to patient survival

Because of sequential expression of the different cyclins during cell cycle we tested for interactions in the Cox regression model. We found a significant interaction between gene amplification of cyclin A and cyclin E (Cox regression, $p = 0.02$). These two cyclins are sequentially time related in the cell cycle. Therefore, effect of amplification of cyclin A gene was tested (i) when cyclin E gene was not amplified and (ii) when cyclin E gene was amplified. When cyclin E gene was not amplified the statistical strength of the cyclin A gene amplification and protein overexpression as well as association to patient survival increased with a HR of 5.5 (95% CI: 2.2–14.3, $p < 0.0001$). When cyclin E gene was amplified, amplification of cyclin A gene had no significant correlation to protein over-expression neither any impact on survival ($p = 0.45$). However, in this subset of patients cyclin A over-expression, as detected by immunehistochemistry, was still significantly related to patient survival (HR 2.8; 95% CI 1.1–7.0; $p = 0.031$).

5. Discussion

In this study we have analysed the impact of gene amplification of both primary (cyclin A, B1, D1, D3 and E) and secondary (cyclin C and H) cyclins on pa-

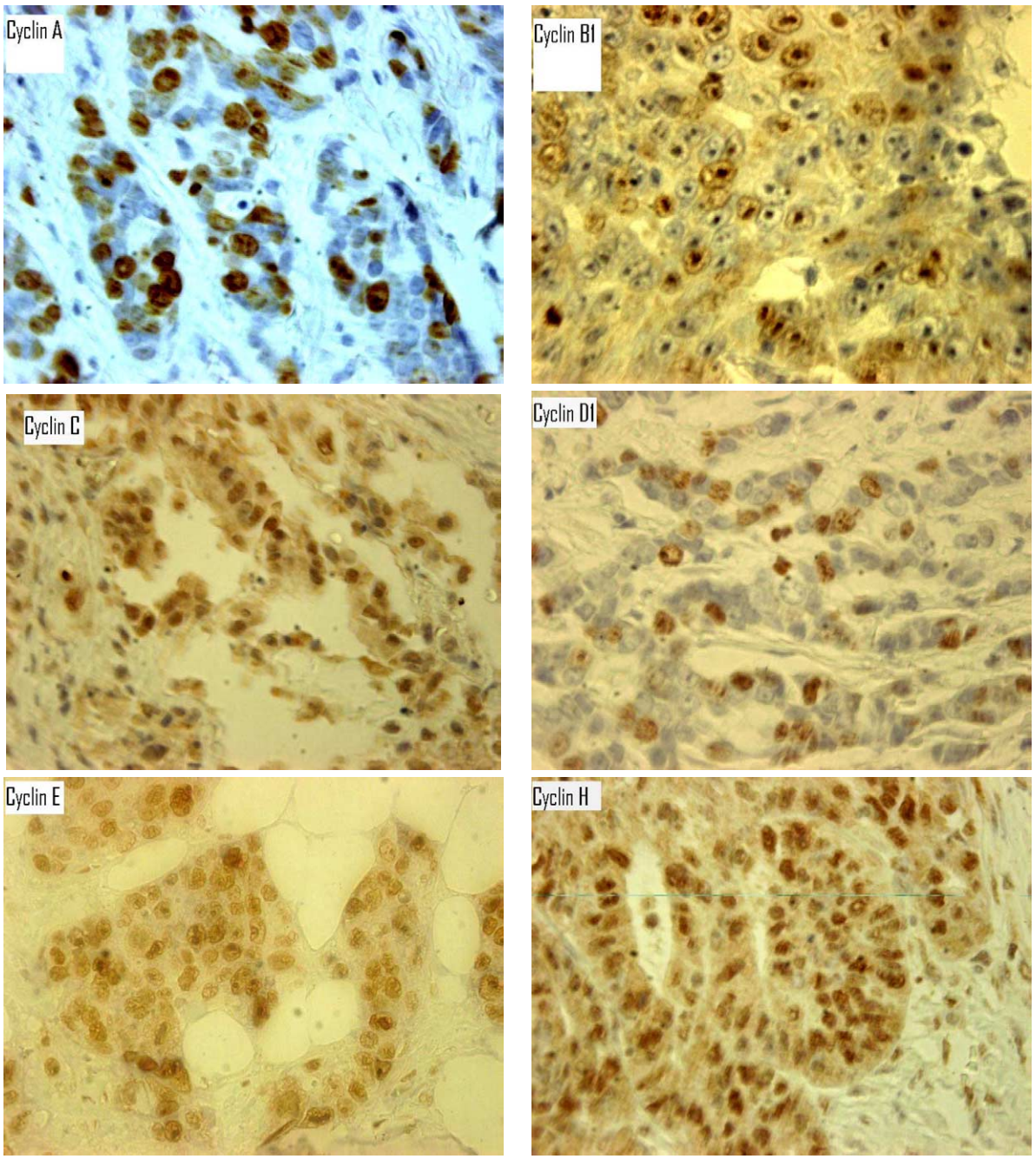

Fig. 1. Examples of immunostaining of different cyclins.

Table 3
Correlation between immunoreactivity and gene amplification among cyclins

	Gene amplification	Protein over-expression	Correlation between gene amplification and protein over-expression	Survival[¤]	Survival[#] protein
Cyclin A	23/80 (26.7%)	65/80 (81.3%)	CO = 0.287, $p = 0.009$	$p = 0.02$ univariate HR = 2.4, $p = 0.032$ multivariate	$p = 0.0031$ univariate HR = 2.3, $p = 0.0004$ multivariate
Cyclin B1	32/79 (37.2%)	47/72 (65.3%)	CO = 0.217, $p = 0.078$	NS	NS
Cyclin C	71/82 (82.6%)	63/75 (86.6%)	0.063	NS	NS
Cyclin D1	64/82 (74.4%)	11/82 (13.4%)	0.977 (0.052)[&]	NS	NS
Cyclin D3	36/86 (41.9%)	54/86 (41.9%)	CO = 0.906, $p < 0.0001$	NS	NS
Cyclin E	25/81 (29.1%)	39/81 (48.1%)	0.410	NS	NS
Cyclin H	8/86 (9.3%)	72/73 (98.3%)	0.932	NS	NS

[¤] Association between gene amplification of different cyclins and patient survival.
[#] Association between over-expression of different cyclins and patient survival.
[&] Adjusted for cyclin A gene amplification.

tient prognosis as well as association between gene amplification and protein expression of these cyclins. We observed that except cyclin A gene amplification, amplification of none of the other cyclins was associated with patient outcome in this cohort. In addition, we observed that the impact of gene amplification of cyclin A on protein over-expression and patient prognosis differed according to amplification status of cyclin E gene. In the group of patients without gene amplification of cyclin E, the gene amplification of cyclin A was associated with over-expression of cyclin A and poor prognosis, while in group of patients with gene amplification of cyclin E, gene amplification of cyclin A had no statistical significant impact on prognosis. Blanchard J.M. has in a review article given evidence for that cyclin E can modulate the expression of cyclin A [4]. In the present study we observed that gene amplification of cyclin E was correlated to over-expression of cyclin A, but not to protein expression of cyclin E. The observation of that no correlation was observed between protein expression of cyclin E and either gene amplification or protein over-expression of cyclin A, may be explained by that the protein over-expression of cyclin E detected by IHC may not be complexed with Cdc2. Only cyclin E-Cdc2 complex is shown to modulate cyclin A expression. It is also possible that degraded protein may have been detected by IHC. Dobashi [11] have in a study demonstrated that there is poor correlation between cyclin E mRNA expression measured by RT-PCR and protein expression measured by IHC, while there was a good correlation between cyclin A mRNA expression and protein expression in lung carcinomas. If this is the case also in breast cancer tumours is difficult to evaluate exactly, since the mRNA measurements have not been performed, but results indicate the same correlation.

We employed real time PCR to detect gene amplification and immunohistochemistry to detect protein expression. Concurrent amplification and over-expression of the respective cyclins varied in our study and support previous findings that protein over-expression is not necessarily caused by gene amplification [5,33]. The mechanisms behind over-expression of cyclins detected in various human malignancies, including breast cancer may be both at gene level, transcription level and posttranscriptional stabilisation of the proteins. The results from this study, as well as some other studies, indicate that the mechanisms behind several of the known proteins may be other than gene amplification. In this study, however, the amplification of cyclin A was the strongest prognostic factor when the preceding cyclin, cyclin E, was not amplified. In this subset amplification of cyclin A was an even stronger prognostic factor than protein expression. When cyclin E was amplified, the prognostic value of cyclin A gene amplification was eliminated, while the cyclin A over-expression as detected by immunohistochemistry, was still significantly associated with cancer death. This indicates that amplification of cyclin A is the strongest factor regulating cyclin A expression in a situation when the preceding cyclin E is "normal" at the genomic level. However, these results indicate that over-expression of cyclin A is a complex process where amplification and expression of both cyclin A and cyclin E can modulate the expression of cyclin A in some tissue. However, correlation between

cyclin A protein over-expression and cyclin E gene amplification has not been demonstrated previously in breast carcinomas.

As has been obvious the last couple of years, aberration in G1/S regulatory proteins are common in various tumours and aberrant expression of cyclin D1, E and A has been observed in several cancers and it can be hypothesized that G1/S defects might be obligatory in tumour development [7,25,36]. However, so far no study has analysed the importance of secondary cyclins on the prognosis of breast cancer patients. The secondary cyclins are involved in pathogenesis of other disease (Alzheimer), and therefore it is of importance to evaluate if secondary cyclins may have any impact in the prognosis of breast cancer patients.

The mechanisms behind the gene amplification are not fully understood. There are some evidence that dysfunction of the p53 may contribute to gene amplification because of genomic instability. Amplifications occur more readily when p53 is inactivated and an amplicon could survive more readily in the absence of normal p53 and thereby accumulate additional amplicons after further cellular divisions [42]. This is also demonstrated in the present study. TP53 gene mutations correlate to gene amplification of primary cyclins such as cyclin A, D3, B1 and D1. However, TP53 gene mutations do not seem to affect the gene amplification status of secondary cyclins, cyclin C and H. It is possible that there is a different mechanism behind gene amplification of primary and secondary cyclins.

Cyclin A over-expression can stimulate re-replication [40]. DNA amplification involves a stretch of DNA much larger than the selected gene that is amplified and co-amplification can occur [37]. The cyclin genes are located on different chromosomes and thus the latter mechanism cannot explain our findings. A non-discriminating mechanism for amplification accumulation like cyclin A over-expression stimulated re-replication could represent a possible underlying mechanism for the enhanced gene copy numbers detected in our study. This hypothesis is further strengthened by the observation that gene amplification and protein over-expression of cyclin A is correlated with gene amplification of other cyclins.

Since there was a strong correlation between gene amplification of different cyclins, we wanted to rule out the possibilities of any methodological artefact. The probe-based homogeneous assay employed in our study is supposed to provide only specific amplification products, since hybridisation of both the primers and the probe is necessary to generate a signal. We also performed real-time quantitative PCR using combinations of primers for one of the cyclins and probes for another cyclin and with appropriate combinations as control in the same run. Only appropriate combinations of primers and probes gave a PCR product.

The frequency of gene amplification of cyclins D1 (79.3%), D3 (39.9 %) and E (87.4%) is higher than those previously reported. None of the previous studies have employed Real time PCR method for detection of gene amplification. Real time PCR is a more sensitive method than for example Southern blotting and Comparative Genomic Hybridisation (CGH) [20] which can be one of the reasons for that we have observed higher frequency of amplified genes. With Real Time PCR it is possible to detect even one additional copy of a gene.

We decided to set a cut off value on level 2. It is difficult to evaluate if doubling of gene copy number is of clinical importance, but it is possible that only one additional copy of some genes may be enough to cause an imbalance in cell cycle regulation.

In our study, the prognostic value of gene amplification of cyclin D1 was changed when adjusted for cyclin A gene amplification. This indicates that cyclin A gene amplification has impact on the effect of cyclin D1 gene amplification. The fact that cyclin D1 gene amplification was associated with better prognosis when adjusted for cyclin A gene amplification can be explained. If the apoptotic function is not impaired, the increased turnover of tumour cells may be associated with better effect of adjuvant therapy and thereby improved relative survival. Increased turnover together with impaired apoptotic function, as a result of cyclin A overexpression, may result in reduced effect of adjuvant therapy, leading to reduced relative survival. Cyclin A over-expression has traditionally not been taken into concern when predicting outcome when cyclin D1 is over-expressed. The prognostic value of cyclin D1 gene amplification and protein over-expression may vary, depending on the distribution of cyclin A over expression in the cohort being studied, and can explain the diverging results between studies of this topic. The impact of cyclin D1 mRNA overexpression has been shown to be different in patients with ER receptor positive tumours compared to patients with ER receptor negative tumours [21]. In our study we did not divide patients into groups according to ER receptor status. A study from van Diest and co-workers have demonstrated that overexpression of cyclin D1 is not associated with patient survival [9]. The results are in accordance with results from the present study when cyclin D1 overexpression was analysed in a univariate analy-

sis of survival, but results are different when analysed in a multivariate analysis of survival. It is difficult to compare results from the present study with previous study, since most of the previous studies have analysed only one or a few of the cyclins at the same time.

In summary, we have analysed gene amplification and protein expression of both primary and secondary cyclins in invasive breast carcinomas. Over expression of cyclin A is correlated to gene amplification of both cyclin A and cyclin E. TP53 gene mutations as well as over-expression and gene amplification of cyclin A is correlated with gene amplification of other cyclins. Only gene amplification and over-expression of cyclin A was associated independently with poor prognosis, and amplification of cyclin A is the strongest prognostic factor in patients that have a normal amplicon of cyclin E.

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