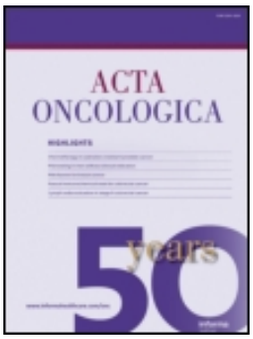

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ORIGINAL ARTICLE

Aberrant expression of cyclin E in low-risk node negative breast cancer

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Abstract

Background. Cyclin E is a cell cycle regulatory protein which occurs in G1, peaks in late G1 and is degraded in early S-phase. Cyclin E overexpression appears to be an independent prognostic factor for overall survival in breast cancer. Nuclear cyclin A is a reliable marker for S-and G2-phases. Consequently, aberrant expression of cyclin E can be detected by simultaneous immunostainings for cyclin A and cyclin E. Studies have shown that aberrant cyclin E might provide additional prognostic information compared to that of cyclin E alone. This study aimed to investigate cyclin E and aberrant cyclin E expression in low-risk node negative breast cancer. *Material and Methods.* We compared women that died from their breast cancer (n = 17) with women free from relapse > 8 years after initial diagnosis (n = 24). All women had stage I, low risk breast cancer. The groups were matched regarding tumour size, receptor status, adjuvant chemotherapy and tumour differentiation. Tumour samples were analysed regarding expression of cyclin A, cyclin E and double-stained tumour cells using immunofloures-cence staining and digital microscopy. *Results.* No differences were seen regarding expression of cyclin E or aberrant cyclin E in cases compared to controls. *Discussion.* We conclude that neither cyclin E nor aberrant cyclin E is a prognostic factor in low-risk node negative breast cancer patients.

Deregulation of the cell cycle is a critical event for the onset of tumorigenesis. Mutations in cell cycle regulatory genes are found in most human cancers [1]. Especially, the cell cycle regulation switching from quiescence to proliferation involving G1-cyclins seems to be defective in cancer [2]. Cyclin E is a cell cycle regulatory protein which is periodically expressed during the cell cycle with protein levels appearing in mid G1, peaking in late G1 and disappearing in early S-phase [3]. Cyclin E has a critical role in G1- to S transition. Anti-cyclin E antibodies injected into fibroblasts during G1 causes cell cycle arrest [4] and, conversely, constitutive cyclin E overexpression leads to shortening of the G1 phase and diminished requirements for growth factors [5]. Furthermore, deregulated cyclin E has also been shown to induce chromosome instability [6].

In normal cells cyclin E expression is rigorously controlled, peaking when necessary and thereafter rapidly degraded. However, in tumour cells regulation of cyclin E expression is often altered. Breast cancer cell lines frequently show amplification of the cyclin E gene and/or constitutively overexpression of cyclin E [7,8]. About 30% of all breast cancer tumours exhibit an abnormal expression of cyclin E [9]. Deregulated cyclin E expression can be caused by several different mechanisms, for example: gene amplification [10], non-cell cycle regulated protein expression [11], tumour-specific alternative splicing of cyclin E into hyper active low-molecular-weight isoforms [12] or defective degradation caused by mutated hCDC4 [13]. Several studies have shown that cyclin E expression correlates to tumour size [14], stage [15], grade [16] and lack of oestrogen receptors [9].

During the past years various clinical studies have examined the role of cyclin E expression as a prognostic factor in breast cancer. High cyclin

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E level has been identified as an independent prognostic factor for poor survival both in node positive and node negative breast cancer regardless if western blot and/or immunohistochemistry was used [15,17,18]. These data were recently confirmed in a meta-analysis reaching the same conclusion [19]. However, in these studies only the question of cyclin E overexpression is addressed giving limited information about true abnormalities in the expression pattern during the cell cycle. Elevated levels of cyclin E could simply be due to increased proliferation in the tumour samples. Recently, an abnormal expression pattern of cyclin E was discovered in tumour cells showing a defect down regulation of cyclin E in early S-phase resulting in aberrantly expressed cyclin E in S-phase and G2-phase [20]. Simplified, the expression pattern of cyclin E was examined by immunoflourescence staining for cyclin E and cyclin A simultaneously, based on a previous paper showing that nuclear cyclin A can be used as a reliable marker for S and G2 phase in both normal and transformed cells [21]. Accordingly, since cyclin E is degraded in early S-phase, cells with a normal cyclin E expression should not co-express cyclin A and E. In a small study on cervical carcinomas, high levels of tumour cells co-expressing cyclin A and were related to a poor outcome [20]. A recently published case-control study on cervical carcinoma confirmed parallel cyclin A and E expression as an indicator for poor prognosis [22].

We wanted to examine tumours from low-risk node negative breast cancer patients to investigate if cyclin E and/or aberrant expression of cyclin E could predict for poor outcome.

Material and methods

Study design

To test our hypothesis we compared women that died early from their breast cancer with women free from relapse >8 years after initial diagnosis. All patients belong to a defined cohort of women diagnosed with breast cancer in the Uppsala-Örebro region 1993–2001. They all had tumours ≤ 2 cm, no lymph node metastases, Elston grade I/II or low proliferation (S-phase), oestrogen- and/or progesterone positive tumours and none had received adjuvant chemotherapy. The patients were identified from our regional breast cancer quality register. Those within the cohort dying from breast cancer are hereafter denoted cases. Women who survived without breast cancer relapse >8 years after initial diagnosis serve as a comparison group, hereafter denoted controls. We planned on having 25 cases and 25 controls. However, since only 34 patients fulfilled the inclusion criteria for controls in the whole region, they were all included. All patients' files and pathology reports were reviewed to validate all data received from the breast cancer quality register. Eight of 25 cases were excluded from the study because of contralateral breast cancer with lymph node metastases or tumour size >2 cm (3 patients), no paraffin blocks were found (2 patients), tumour size >2 cm (1 patient), distant metastases at diagnosis (1 patient) and non breast cancer death (1 patient). Ten of 34 controls were excluded from the study because of diagnosis or death from a concurrent cancer (3 patients), relapse or death in breast cancer (3 patients), no paraffin blocks were found (3 patients) and having lymph node metastasis (1 patient). Remaining in the study after reviewing the data were 17 cases and 24 controls.

Patients

See Table I for summary of patient characteristics and adjuvant systemic treatment.

Tumour sizes were comparable in both groups. All tumours were oestrogen- and/or progesterone receptor positive. Six of the cases and 3 of the controls had progesterone receptor negative tumours. One of the inclusion criteria was Elston grade I/II, however, our board certified pathologist re-graded all tumours and found that some were grade III-tumours. Mean Elston points in cases was 6.8 and in controls 6.1 (non-significant difference), qualifying both groups as grade II tumours in average. No tumours were excluded because of differences in re-grading. Most tumours were of ductal histology: 16 ductal and 1 lobular carcinoma in the case group and 20 ductal, 3 lobular and 1 mucinous carcinoma in the control group. None of the patients received adjuvant chemotherapy. However, 11 cases and 20 controls who were surgically treated with sector resection received adjuvant radiotherapy. Three cases received adjuvant antihormonal treatment versus none of the controls. Mean time to distant metastases and survival among cases was 23 months and 43 months, respectively. Mean follow-up among controls was 139 months.

Immunoflourescence stainings

Immunoflourescence stainings were performed on paraffin-embedded tissue sections from breast cancer tumours. Four-five μm thick sections were cut from the paraffin-blocks and mounted on super-frost slides. The sections were deparaffinised and thereafter rehydrated through a ladder of graded ethanol (absolute ethanol, 90%, 70%, 50% and 30%). An antigen retriever (2100 Retriever, PickCell Laboratories, Amsterdam, NL) was used to recover the

Table I. Patient characteristics and staining results.

	T (mm)	Elston Grade	PgR	Adjuvant RT	Adjuvant ET	Follow-up (months)	Cyclin A (%)	Cyclin E (%)	DB (%)	Fraction DB (%)
Node negative patients										
Cases	13	I	−	yes	no	60	7	5	1.8	24
	18	II	+	no	no	66	11	5	0.5	4
	10	II	+	no	yes	32	9	1	0.3	4
	17	III	+	yes	no	41	25	6	3.2	13
	17	II	+	no	no	91	6	6	1.2	21
	12	III	−	yes	no	12	7	12	1.3	18
	15	I	−	no	no	16	14	0	0.0	0
	17	II	+	yes	no	44	7	8	1.4	21
	17	II	−	yes	yes	19	10	10	2.8	27
	10	III	−	yes	no	21	11	4	0.4	2
	12	II	+	yes	no	45	7	6	1.5	21
	17	III	+	yes	yes	20	10	2	0.9	9
	14	III	+	yes	no	16	9	4	0.9	10
	17	II	+	yes	no	67	35	7	5.1	15
	3	II	+	yes	no	99	1	1	0.0	0
	12	III	−	no	no	36	4	1	0.2	5
	12	II	+	no	no	47	6	3	0.5	8
Mean	15	II				43	11	5	1.3	12
Controls	15	I	+	no	no	152	25	4	3.3	14
	7	III	+	yes	no	136	2	4	0.2	10
	10	II	−	yes	no	145	20	8	6.3	31
	16	II	+	yes	no	148	4	3	0.1	3
	10	II	+	yes	no	158	13	1	0.5	4
	6	II	+	no	no	148	13	5	3.5	27
	16	II	+	yes	no	145	18	4	1.6	9
	14	III	+	yes	no	137	4	2	0.2	5
	19	II	−	yes	no	125	7	6	0.6	8
	16	III	+	yes	no	144	7	5	1.2	16
	9	I	+	yes	no	146	6	5	1.3	21
	6	III	+	yes	no	130	4	1	0.0	0
	14	II	+	yes	no	122	20	7	2.6	13
	5	I	+	yes	no	147	16	1	0.4	3
	17	II	+	no	no	133	6	6	0.8	13
	13	II	−	yes	no	123	12	1	0.4	3
	10	II	+	yes	no	132	20	2	0.7	3
	7	I	+	yes	no	138	3	2	0.1	5
	10	I	+	no	no	122	6	4	0.3	5
	11	II	+	yes	no	133	12	4	1.6	14
	13	I	+	yes	no	149	4	4	1.5	38
	12	II	+	yes	no	133	21	7	5.3	25
	11	I	+	yes	no	133	18	12	7.6	42
	10	II	+	yes	no	159	30	11	7.9	26
Mean	12	II				139	12	5	2.0	14

All tumours are T1, N0 and stain positive for oestrogen and/or progesteron.
T = tumor size, PgR = progesteron receptors, + = positive, − = negative, RT = radiotherapy, ET = endocrine treatment, Follow-up = survival in cases, DB = doublestained, fraction DB = fraction doublestained cells in S-phase.

tissue sections' antigenicity by heating the slides in citrate acid (pH6) to 120C for 20 minutes and thereafter slowly cooling them in room temperature during 2 hours. Blocking buffer (1% bovine serum albumin and 0.5% Tween 20 dissolved in phosphate-buffered saline) was applied for 10 minutes. A polyclonal rabbit antibody (H432; Santa Cruz Biotechnology, Santa Cruz, USA) was used to detect cyclin A and a monoclonal mouse antibody (H12; BD Pharmingen, San Diego, CA, USA) to detect cyclin E, both applied for overnight incubation. Three washing steps, 20 minutes each, were performed in washing buffer. Unspecific binding of the secondary antibodies was blocked by incubation of the slides in 4% donkey serum diluted in blocking buffer. Secondary antibodies, goat anti-rabbit Cy5

(ab 6564-100, Abcam, Cambridge, UK) and donkey anti-mouse biotinylated (00007754; Dako Cytomation, Carpenteria, CA, USA), were added to the slides and incubated in room-temperature for 2 hours. An amplification step with biotin and streptavidin was used to enhance the signal from cyclin E. Visualisation of cyclin E was accomplished by adding Streptavidin-Cy3 (PA 43001, Amersham Life Sciences) binding to the biotinylated secondary antibody. Cover slips were mounted for fluorescence microscopy in mounting medium (Vectashield, Vector Laboratories Inc, Burlingame, CA, USA) containing DAPI (2–4-amidinophenyl-6-indolecarbamidine dihydrochloride). As positive control we used tonsil and as negative control omission of primary antibodies. This staining protocol resulted in cell nuclei stained with DAPI, cyclin A stained with Cy5 and cyclin E with Cy3.

Image acquisition and analysis

Ten images were acquired from each tumour slide using a Delta Vision system (Applied Precision Inc, Issaqua, WA, USA) equipped with a monochrome water-cooled CCD camera (Photometrics Ltd, Tucson, AZ, USA). We used a Plan-Neofluar 63x/NA 1.40 lens which resulted in images with a resolution of 0.2 μm. Each image acquisition involved taking three photos with optical filters detecting DAPI, Cy3 and Cy5. Image analysis was performed using the IMP processing software. A minimum of 500 cells were counted on each tissue section. Only stainings arising from cell nuclei were considered as truly positive. Since cyclin E normally is degraded early in S-phase (when cell nuclei are weakly positive for cyclin A) and we were focusing mainly on aberrant cyclin E expression, only cell nuclei staining moderately or strongly positive for cyclin A (representing cells in S-phase and G2-phase) were counted as positive. In order to determine which stainings were to be considered positive we manually set thresholds by visual inspections of the images.

Statistics

The study was designed as a comparison between two groups of women with different outcome status – early death from breast cancer versus survival without recurrence more than 8 years – sampled from one defined cohort. We denote our groups cases and controls, although they are not sampled according to the classical case-control design. Sample size was calculated using Lehr′s formula: to detect a minimum difference in the distribution of a continuous variable of one standard deviation 16 patients ($\alpha = 0.05$; $\beta = 0.20$) or 21 patients ($\alpha = 0.05$; $\beta = 0.10$) were needed in each group. Comparisons between cases and controls were performed using Student t-test (double-sided). Differences were considered statistically significant if $p < 0.05$.

Results

See Table I for summary of staining results and Figures 1 and 2.

Tumours sections from cases stained positive for cyclin A in 11% (95% confidence interval [95 CI]: 7–14%) and for cyclin E in 5% (95 CI: 3–6%) of all

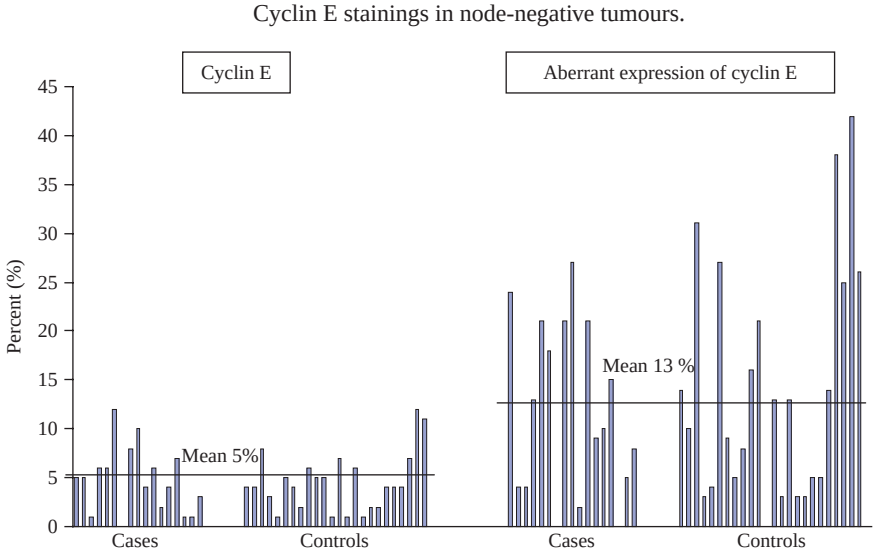

Figure 1. Diagrams showing percent of all tumour cells staining positive for cyclin E and aberrant expression of cyclin E, i.e. fraction of cells co-expressing cyclins A and E, in cases and controls with node negative breast cancer.

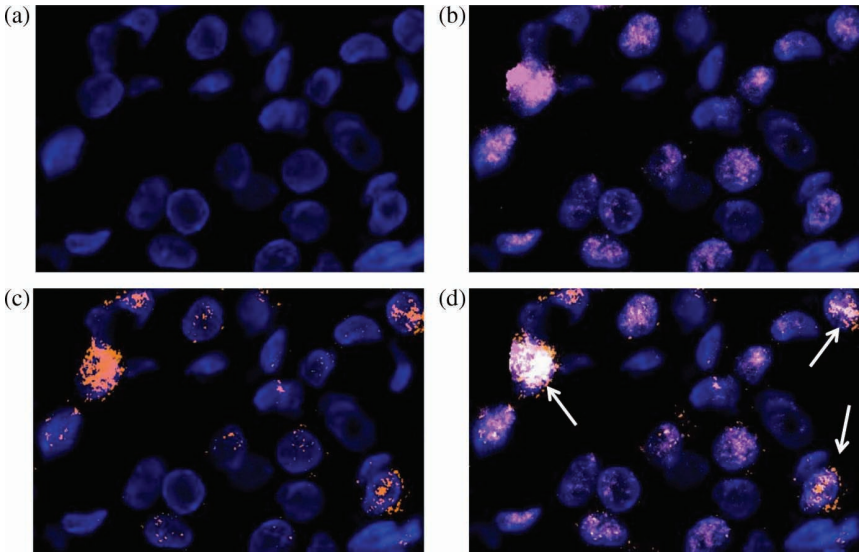

Figure 2. Photos illustrating a. cell nuclei stained with DAPI, b. cyclin A positive cells (Cy 5), c. cyclin E positive cells (Cy 3) and d. cells co-expressing cyclins A and E (arrows).

tumour cells. Co-expression of both cyclin A and E occurred in 1.3% (95 CI: 0.7–1.9%) of the tumour cells. Fraction of double-stained cells in S-phase, calculated as cells co-expressing cyclin A and E divided by cells staining positive for cyclin A, was 12% (95 CI: 8–16%).

The corresponding figures for control patients were 12% (95 CI: 9–15%) for positive cyclin A staining, 5% (95 CI: 3–6%) for positive cyclin E staining and 2.0% (95 CI: 1.0–3.0%) for positive co-expression of both cyclins A and E. Fraction of double-stained cells in S-phase was 14% (95 CI: 9–19%).

No statistically significant differences regarding cyclin A expression (2%, 95 CI: -3 to 6%), cyclin E expression (0%, 95 CI: −2 to 2%), co-expression (0.7%, 95 CI: −0.5 to 1.9) or fraction double-stained cells (2%, 95 CI: −4 to 8%) were seen between cases and controls.

Discussion

This study on low-risk node negative breast cancer patients could not detect any differences regarding expression of cyclin A, cyclin E or aberrant expression of cyclin E comparing cases to controls. Mean values were very similar in the subgroups with a mean value for cyclin A expression of 11% in cases and 12% in controls, for cyclin E expression of 5% both in cases and controls and for aberrant cyclin E expression of 12% and 14%, respectively (Table I and Figure 1).

Our results generally show lower levels of positive cells than previously published studies. Kühling et al. [17] reported a mean of 9.5% (range 0–90%) cyclin E positive cells in node negative patients and Bukholm et al. [23] reported that 40% of tumours from node positive and node negative patients stained positive for cyclin E in >15% of the cells. This should be compared to our results in node negative tumours, mean 5% (range 0–12%). One probable explanation could be that all patients in our study had seemingly excellent prognosis with small tumours, node negativity, receptor positivity and none having received adjuvant chemotherapy. Kühling et al. and Bukholm et al. had mixed patient groups participating in their studies. In Kühling et al.'s study half of the patients had tumours larger than 2 cm and one third of them had receptor negative disease and in Bukholm et al.'s study half of the patients had node positive disease. It is well known that cyclin E expression increases with tumour size, grade, stage and lack of hormone receptors and, consequently, our results make sense and should have been expected [9,14–16].

We deliberately chose to investigate a patient group with node negative tumours having seemingly excellent prognostic outcome, consisting of patients dying early from breast cancer despite good odds. As a comparison group we used another extreme: women surviving more than 8 years without a relapse. We thus excluded women that have a short follow-up time and were censored before 8 years of follow-up because they are non-informative regarding their long-term natural history. This comparison of two extreme groups should theoretically have detected any reasonably sensitive and specific marker for prognosis. These patients are considered having such good prognosis that they are not

offered adjuvant chemotherapy according to the local treatment recommendations. Still, some of them relapse and might have benefited from such adjuvant treatment. However, this study shows that assessing cyclin E does not help us separate these two patient groups with different prognosis from each other.

In a small pilot study we examined cyclin E and aberrant cyclin E in high-risk node positive breast cancer patients. We used the described staining protocol on 5 tumours from patients dying early from breast cancer and 10 tumours from patients still alive 7 years after diagnosis. We found that non-survivors stained significantly more often positive for cyclin E (15% versus 4%, $p < 0.01$), co-expression of both cyclins (7.0% versus 1.5%, $p < 0.01$) and fraction of double-stained cells in S-phase (22% versus 6%, $p < 0.01$) than survivors. Preliminary data indicate that cyclin E and aberrant expression of cyclin E might separate survivors from non-survivors in this patient group encouraging us to perform a larger study on high-risk node positive breast cancer patients.

We conclude that neither cyclin E nor aberrant cyclin E is a prognostic factor in low-risk node negative breast cancer. However, we believe that the role of cyclins in worse-prognosis node negative patients is not yet fully looked into. Since our study covered only a more defined set of breast cancers with certain histopathological characteristics and was limited to stage I, we cannot exclude that expression of cyclin A and E would help distinguish different prognostic groups in settings with a broader spectrum of breast cancers. Presently, we are performing a larger case-control study also including node negative patients with worse prognosis i.e. patients with larger and receptor negative tumours, to further investigate the role of cyclins A, B, D and E as prognostic factors in node negative breast cancer patients.

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