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ORIGINAL ARTICLE

Cyclin B is an immunohistochemical proliferation marker which can predict for breast cancer death in low-risk node negative breast cancer

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Abstract

Patients with low-risk node negative breast cancer have an excellent prognosis with 5% breast cancer mortality at 10 years. However, prognostic factors are needed to identify poor prognostic patients who might benefit from adjuvant systemic therapy. Proliferation has been identified as the most important component of gene expression profiles. Cyclin B is a proliferative marker easily assessed by immunohistochemistry. We wanted to examine cyclin B as a prognostic factor in low-risk breast cancer patients. *Patients and methods*. Using an experimental study design, we compared women dying early from their breast cancer (n=17) with women free from relapse more than eight years after initial diagnosis (n=24). All women had stage I, node negative and hormone receptor positive disease. None had received adjuvant chemotherapy. Tumor samples were immunostained for cyclin B using commercial antibodies. *Results*. The mean percentage of cyclin B (12%) was significantly higher (p=0.001) in women dying from their breast cancer compared with women free from relapse (5%). High cyclin B (≥9%) identified 11/17 patients dying from breast cancer and low cyclin B identified 22/24 patients free from relapse. The sensitivity and specificity of cyclin B was 65% and 92%, respectively. *Discussion*. We found that low-risk node negative patients with high expression of cylin B had a significantly worse outcome than patients with low expression of cyclin B. Cyclin B could separate patients with poor survival from those with good survival with 80% accuracy. We suggest that cyclin B might be a potent prognostic factor in this low-risk patient group.

There is concern regarding overtreatment with chemotherapy in patients with node negative breast cancer. Based on established prognostic factors such as tumor size, hormone receptors, histologic grade and overexpression of HER2 many node negative breast cancer patients are offered adjuvant chemotherapy even though the absolute survival benefit could be as low as 5% [1–3]. This is largely due to our inability to identify patients unlikely to benefit from such treatment. However, we also need to consider the possibility of undertreatment. A subgroup of node negative breast cancer with stage I disease and hormone receptor positivity has an excellent prognosis with a breast cancer mortality of only 5% at 10 years. However, this means that 5% of these patients have a poor prognosis and might have benefited from adjuvant systemic therapy. Again, the problem is finding a tool to identify these patients.

Most studies agree that proliferation markers such as Ki-67 and cyclins are important prognostic tools in breast cancer [4]. However, proliferation markers are not recommended for routine clinical use due to lack of standardization of staining procedures and scoring [5,6]. Recently, proliferation has been identified as the driving force of modern gene expression profiles [7]. This further highlights the need to find an accurate immunohistochemical marker for proliferation which can easily be incorporated into routine clinical work.

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Cyclin B plays an essential role as a mitotic cyclin in the entry of mitosis from the G2 phase [8]. Several studies have shown that cyclin B is a proliferative marker with ability to predict for early relapse and a poor outcome in early breast cancer [9–11]. We examined if cyclin B could separate patients with a poor outcome from those with a good outcome in a subgroup of patients with low-risk node negative breast cancer.

Patients and methods

Study design

We compared women that died early from their breast cancer with women free from relapse more than eight 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 tumors ≤ 2 cm, no lymph node metastases, histologic grade I/II or low proliferation (S-phase), estrogen (ER) and/or progesterone (PgR) receptor positive tumors and none had received adjuvant chemotherapy. The patient cohort was identified from a regional breast cancer register set up for clinical audit and research. The register is matched to the by-law mandatory cancer registration and the population coverage is 99% [12]. Those within the cohort dying from breast cancer are hereafter denoted cases. Women who survived without breast cancer relapse more than eight years after initial diagnosis serve as a comparison group, hereafter denoted controls. We planned on having 25 cases and 25 controls. As cases we chose the 25 women with the shortest survival after their breast cancer diagnosis. Only 34 patients fulfilled the inclusion criteria as controls and all were 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 tumor size >2 cm (three patients), no paraffin blocks were found (two patients), tumor size >2 cm (one patient), distant metastases at diagnosis (one patient) and non breast cancer death (one patient). Ten of 34 controls were excluded from the study because of diagnosis or death from a concurrent cancer (three patients), relapse or death in breast cancer (three patients), no paraffin blocks were found (three patients) and having lymph node metastasis (one patient). Remaining in the study after data review were 17 cases and 24 controls.

Patients

Table I shows patient characteristics and their adjuvant systemic treatment. Tumor sizes were comparable in both groups. All tumors were ER and/or PgR positive. Six of the cases and three of the controls had PgR negative tumors. One of the inclusion criteria was histologic grade I/II, however, our board certified pathologist regraded all tumors and found that some were grade III-tumors. Mean Elston points in cases was 6.8 and in controls 6.1 ($p>0.5$), qualifying both groups as grade II tumors in average. No tumors were excluded because of differences in regrading. There were 16 ductal and one lobular carcinoma in the case group and 20 ductal, three lobular and one 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 breast cancer specific survival among cases was 23 months and 43 months, respectively. Mean follow-up among controls was 139 months.

Immunohistochemical stainings

Immunostainings were performed on paraffin-embedded tissue sections from breast cancer tumors. Four to 5 μm thick sections were cut from the paraffin-blocks and mounted on super-frost slides. Conventional slides were deparaffinized in xylene and rehydrated through a ladder of graded ethanol (absolute ethanol, 95%, 80% and distilled water). Antigen retrieval was done in tris buffer (pH=8) using a pressure cooker before processing the sections in an automatic immunohistochemistry staining machine according to standard procedures (Autostainer; Dako, Sweden). The primary antibody for detection of cyclin B1 (rabbit monoclonal; Y106; Epitomics Inc, Burlingame, CA, USA) was applied for 30 minutes at room temperature. Immunostainings were detected via DAKO Cytomation envision/HRP kit K5007. Tonsil samples were used as positive controls. The primary antibody was omitted from negative controls.

In most tumors 1 000 cells were counted, with a minimum level set at 200 cells. Nuclear as well as cytoplasmic stainings were assessed in a comparative study. Cut-off value separating high and low cyclin B_{total} (total=nuclear and/or cytoplasmic staining) was set at ≥ 9% corresponding to the 7th decile of all patients' values.

Both staining and scoring were done blinded to case and control status.

Statistics

The study was designed as a comparison between two groups of women with different outcome status – early

Table I. Patient characteristics and staining results.

		T (mm)	Grade	Adjuvant RT	Adjuvant ET	Follow-up (months)	Cyclin $B_{nuclear}$ (%)	Cyclin B_{total} (%)
Node negative patients	Cases	13	I	yes	no	60	0	4
		18	II	no	no	66	20	20
		10	II	no	yes	32	1	6
		17	III	yes	no	41	1	11
		17	II	no	no	91	0	5
		12	III	yes	no	12	1	16
		15	I	no	no	16	2	11
		17	II	yes	no	44	0	6
		17	II	yes	yes	19	0	9
		10	III	yes	no	21	0	25
		12	II	yes	no	45	1	10
		17	III	yes	yes	20	0	16
		14	III	yes	no	16	1	21
		17	II	yes	no	67	2	5
		3	II	yes	no	99	0	4
		12	III	no	no	36	2	26
		12	II	no	no	47	0	12
	Mean	**15**	**II**			**43 (12–99)**	**2**	**12**
	Controls	15	I	no	no	152	0	3
		7	III	yes	no	136	0	8
		10	II	yes	no	145	0	0
		16	II	yes	no	148	1	5
		10	II	yes	no	158	1	3
		6	II	no	no	148	0	6
		16	II	yes	no	145	1	4
		14	III	yes	no	137	0	4
		19	II	yes	no	125	1	10
		16	III	yes	no	144	0	13
		9	I	yes	no	146	7	4
		6	III	yes	no	130	0	6
		14	II	yes	no	122	0	2
		5	I	yes	no	147	0	6
		17	II	no	no	133	0	8
		13	II	yes	no	123	0	0
		10	II	yes	no	132	1	7
		7	I	yes	no	138	0	4
		10	I	no	no	122	0	7
		11	II	yes	no	133	0	2
		13	I	yes	no	149	0	4
		12	II	yes	no	133	0	8
		11	I	yes	no	133	0	6
		10	II	yes	no	159	0	2
	Mean	**12**	**II**			**139 (122–159)**	**0.5**	**5**

T=tumor size, ET=endocrine treatment, RT=radiotherapy, Follow-up=survival in cases, Cyclin $B_{nuclear}$=nuclear cyclin B, Cyclin B_{total}=total (nuclear and/or cytosplasmic staining).

death from breast cancer versus survival without recurrence more than eight 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. Since the patients were selected, Fisher's exact test was used comparing cases and controls. For comparison of means Mann-Whitney was used. Differences were considered statistically significant if $p<0.05$.

Results

See Table I for summary of staining results. Tumor sections from cases stained positive for cyclin B_{total} in 12% (95% confidence interval [95 CI]: 8–16%) and for cyclin $B_{nuclear}$ (nuclear staining only) in 2% (95 CI: 0–5%) of all tumor cells. The corresponding figures for control patients were 5% (95 CI: 4–6%) for cyclin B_{total} staining and 0.5% (95 CI: 0–1%) for cyclin $B_{nuclear}$.

There was a statistically significant difference between mean cyclin B in cases and controls, both regarding cyclin B_{total} (12% and 5%, p=0.001) and cyclin $B_{nuclear}$ (2% and 0.5%, p=0.05).

High cyclin B_{total} (≥ 9%) could accurately identify 11 of 17 patients dying from breast cancer and low cyclin B_{total} could accurately identify 22 of 24 patients free from relapse eight years after initial diagnosis. This yields a sensitivity of 65% (95 CI: 38–86%) and specificity of 92% (95 CI: 73–99%), corresponding to a positive and negative likelihood ratio of 7.8 and 0.38 respectively. Figure 1 shows the ROC curve for cyclin B_{total}. The overall accuracy for identifying patients dying from breast cancer and long time survivors using cyclin B_{total} was 80% (33/41). The corresponding accuracy for cyclin $B_{nuclear}$ was 66% (27/41) using cut-off ≥1%.

Discussion

In this study, we have shown that assessing cyclin B expression in tumors can help separate patients with a poor outcome from those with a good outcome in a subgroup of patients with low-risk node negative breast cancer. We deliberately chose to investigate a group of women with node negative tumors and other favorable tumor characteristics, yet dying early from breast cancer despite a seemingly excellent prognosis. As a comparison group we used another extreme: women surviving more than eight years without a relapse. We thus excluded women that have a short follow-up time and were censored before eight years of follow-up because they are non-informative regarding their long-term natural history. The women in our case group are considered having such a good prognosis that they are not offered adjuvant chemotherapy according to national treatment recommendations.

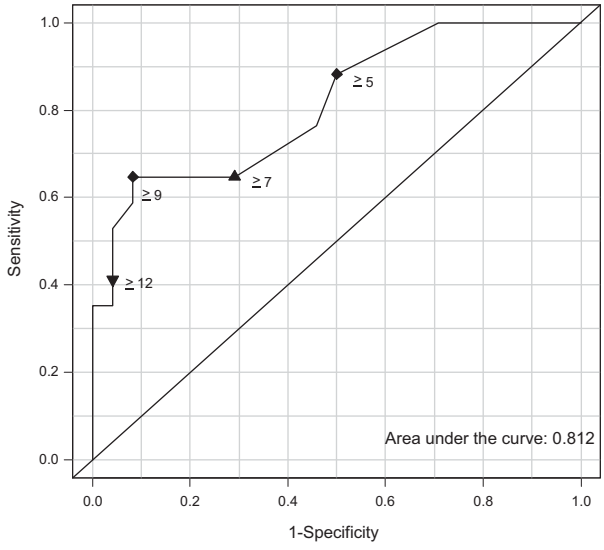

Figure 1. ROC curve for cyclin Btotal. Different cut-off values are presented in the figure.

Still, some of them relapse and might have benefited from such adjuvant treatment.

Our study population is recruited from a population-based database with near complete coverage and no losses to follow-up. Thus, the study does not suffer from referral bias and we have a valid establishment of end-point. We excluded women alive but with a follow-up of less than eight years, since these women would not be truly informative regarding a long-term good prognosis. However, since we included all breast cancer deaths in the observed period, this should not bias our results as if we had only included women dying early in the case group. We may, however, have some overrepresentation of women with early deaths given the long natural history of breast cancer. The major limitation of the study is the limited sample size.

In most previous papers examining the prognostic ability of cyclin B the authors have looked at cyclin B_{total} (cytoplasmic and/or nuclear cyclin B) [9,10]. However, Suzuki et al. claims that only nuclear cyclin B is a prognostic factor in breast cancer [11]. Thus, we examined both cyclin $B_{nuclear}$ (nuclear cyclin B) and cyclin B_{total} and found that both were significantly higher in patients dying from breast cancer compared to long time survivors. However, the difference between the two groups was greater using cyclin B_{total} than cyclin $B_{nuclear}$ (12–5% and 2–0.5%, respectively), making cyclin $B_{nuclear}$ impractical in the clinical setting. Also, the accuracy of correctly identifying patients dying from breast cancer and survivors was higher using cyclin B_{total} (80% and 66%, respectively). Since our interest lies in finding new prognostic factors that can be implemented in the clinical situation no further analyzes were made on cyclin $B_{nuclear}$ and the rest of the discussion focuses on cyclin B_{total}.

Our results – that a proliferation marker can separate patients with a poor outcome from those with a good outcome – are well in line with previous studies showing that gene expression signatures' prognostic abilities are due mostly to the detection of proliferation activity [7,13,14]. Furthermore, recent data suggest that their performance is limited to the ER+/HER2- subgroup. Thomassen et al. compared the performance of several gene sets including, amongst others, the 70-gene and the 76-gene signature to predict for metastases in low-malignant breast cancer. The accuracy to correctly predict for metastases varied from 48–75%. The authors also tested their 32-gene profile (HUMAC32) which was previously developed from this data set and found that their accuracy was 78%. This should be compared to the accuracy achieved in our study when using immunohistochemically assessed cyclin B, i.e. 80%. Of course, a direct comparison cannot be performed since we have not used the same patient material. However, we both have used subgroups of patients

with low-malignant breast cancer not having received adjuvant chemotherapy in which we have been able to identify poor-prognostic patients with high accuracy. Those poor prognostic patients identified as having highly proliferative tumors might very well benefit from adjuvant chemotherapy.

Since the overall accuracy does not illustrate the overlap between cases and controls and thus only indirectly is informative for the possible clinical uses of the test, we show sensitivity, specificity and likelihood ratios. The results indicate that the proportion of false positives (1-specificity=8%), i.e. those overtreated, would be acceptable, but the proportion of false negatives (1-sensitivity) would be around 35% and missed opportunities thus potentially too high. However, to decide the utility of the test, we would also have to know more about the response rate in the false negatives. Furthermore, the test can potentially be clinically very helpful combined with a more sensitive test that could minimize the risk of undertreatment.

No matter how promising and interesting gene profiling might be, there are many oncologists today that cannot offer their patients gene expression profiling because of low availability and poor economy. Within a decade, a majority of breast cancer patients will live in countries with sparse resources. Furthermore, most studies in the field have not hitherto analyzed the tests from a standpoint of classification. Our results show that sensitivity, specificity and likelihood ratios need to be considered together with overall accuracy to understand the potential use of the test. Even so, a decision analysis taking positive and negative effects of the decision following the test is needed before the test is fully implemented. Until these prognostic signatures are readily available at a reasonable cost most oncologists have to rely on immunohistochemical proliferation markers. We need to further improve standardization of methodology regarding staining procedures, scoring and cut-off values of immunohistochemical markers.

We conclude that cyclin B which is easily assessable using immunohistochemistry potentially can be clinically useful to predict for breast cancer death in a subgroup of low-malignant breast cancer patients with high specificity. However, one should be aware that our study-design may over-estimate the results compared to a regular cohort study, but the design implies that we may not have correctly classified women with risk of dying very late from their breast cancer. Nevertheless, our promising results motivate for validating studies.

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