Detection of Low Molecular Weight Derivatives of Cyclin E1 Is a Function of Cyclin E1 Protein Levels in Breast Cancer

Charles Spruck, Dahui Sun, Heidi Fiegler, Christian Marth, Elisabeth Mueller-Holzner, Georg Goebel, Martin Widschwendter, and Steven I. Reed

Abstract
Cyclin E1 regulates the initiation of the S phase program in the mammalian cell division cycle. In normal cells, cyclin E1 protein expression is tightly controlled through a combination of transcriptional and proteolytic regulatory processes. However, in many types of human tumor, cyclin E1 expression is frequently dysregulated, including overexpression, nonperiodic expression relative to cell division, and generation of low molecular weight (LMW) derivatives. LMW derivatives of cyclin E1 have been proposed to be generated by the in vivo proteolytic cleavage of the full-length cyclin E1 protein by a yet to be identified tumor-specific protease. Recently, it was suggested that overexpression of full-length or LMW derivatives of cyclin E1 are independent variables associated with poor outcome in patients with breast cancer. However, we have extensively analyzed cyclin E1 protein expression in primary breast tumors and breast tumor-derived cell lines and found that the ability to detect LMW derivatives of cyclin E1 correlates only with the level of cyclin E1 protein. When cyclin E1 levels on Western blots are normalized, LMW derivatives of cyclin E1 were observed at roughly equal levels in all primary breast tumors, breast tumor-derived cell lines, immortalized nontransformed human mammary epithelial cells, and normal breast tissue. Therefore, the detection of LMW derivatives of cyclin E1 is likely a function of cyclin E1 protein levels, and the activity of the proteolytic machinery responsible for their generation is not a tumor-specific property. (Cancer Res 2006; 66(14): 7355-60)

Introduction
Cyclin E1, an activator of cyclin-dependent kinase (Cdk) 2, regulates the G1-S phase transition of the mammalian cell division cycle (1–3). Together with the D-type cyclins (D1, D2, and D3), the E-type cyclins (E1 and E2) function by integrating positive growth cycle (1–3). Together with the D-type cyclins (D1, D2, and D3), the E-type cyclins (E1 and E2) function by integrating positive growth.

Note: Current address for C. Spruck: Department of Tumor Cell Biology, Sidney Kimmel Cancer Center, 10835 Road to the Cure, San Diego, CA 92121. Current address for M. Widschwendter: Department of Gynaecological Oncology, University College London, London, WCIE 0H, United Kingdom.

Requests for reprints: Steven I. Reed, Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. Phone: 858-784-9836; Fax: 858-784-2781; E-mail: sreed@scripps.edu.

©2006 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-05-3240


Downloaded from cancerres.aacrjournals.org on May 1, 2017. © 2006 American Association for Cancer Research.
Finally, we show that the proteolytic machinery responsible for the cleavage of full-length cyclin E1 into LMW derivatives is present in nontransformed mammary epithelial cells and normal breast tissue, contrary to what has been proposed previously (22, 27).

**Materials and Methods**

**Tissue samples.** Breast cancer specimens (N = 138) were obtained immediately after resection of the breast or lumpectomy at the Department of Obstetrics and Gynecology, Innsbruck Medical University (Innsbruck, Austria). Specimens were brought immediately to a pathologist, and a portion of the tissue was placed into liquid nitrogen and then stored long term at −80°C. Of the 138 breast cancer patients, 35 (25.4%) were diagnosed with pT1N0, 80 (58%) with pT2N0, and 19 (13.8%) with pT3N0 (information for 4 patients were missing). Of the 54 (39.1%) patients nodal negative, and 82 (60.9%) patients were nodal positive (information for 2 patients were missing). These included 101 (73.2%) invasive ductal carcinomas, 18 (13%) lobular carcinomas, and 18 (13%) carcinomas otherwise differentiated (medullary, mucinous, papillary, and tubular carcinomas; information for 1 patient were missing). Estrogen and progesterone receptor status were identified immunohistochemically and/or biochemically. Eighty-nine (64.5%) specimens were hormone receptor positive (defined as estrogen receptor and/or progesterone receptor positive), whereas 46 (33.3%) were hormone receptor negative. The mean age at diagnosis was 61.8 years. Thirty-eight (27.3%) were premenopausal and 99 (71.7%) were postmenopausal.

**Western blot analysis.** Extracts were prepared by lysis of tumor specimens in mammalian radioimmunoprecipitation assay buffer (RIPA; 150 mmol/L NaCl, 50 mmol/L Tris (pH 8), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mmol/L NaF, 1 mmol/L orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin). Samples were incubated on ice for 5 minutes and sonicated briefly, and extracts were clarified by centrifugation at 16,000 g for 15 minutes at 4°C. For comparisons of extract preparation techniques, procedures used were identical to two different extract preparation techniques referenced by Keyomarsi et al. (10, 27, 28). For Western blot analysis, total protein (50 μg) was separated on 10% SDS-PAGE gels, blotted onto Immobilon-P membranes (Millipore, Bedford, MA), and hybridized with anti-cyclin E1 antibody HE12 (29), which was used previously to analyze LMW forms of cyclin E1 (10). Blots were exposed to X-ray film for varying lengths of time: shorter exposures to detect full-length cyclin E1 protein in a linear exposure range and longer exposures to detect LMW forms. Cyclin E1 protein level was scored on a scale of 1 (low) to 4 (very high) and LMW forms on a scale of 1 (low/absent) to 4 (very high), with pT1, 80 (58%) with pT2, and 19 (13.8%) with pT3N0 (information for 4 patients were missing).

**Results**

**Overexpression of full-length and LMW derivatives of cyclin E1 in primary breast tumors.** We investigated cyclin E1 protein expression in 138 randomly chosen primary breast tumor specimens by Western blot analysis using monoclonal antibody HE12 (29), which recognizes both full-length and LMW forms of cyclin E1 and was used in previous studies of LMW derivatives. Cyclin E1 protein expression was observed in all tumors analyzed with a predominant band migrating at ~50 kDa corresponding to the full-length cyclin E1 protein (Fig. 1A). Thirty (22%) tumors were found to express high or very high levels of cyclin E1 protein, a comparable frequency to that observed in previous studies (10). Statistical analysis revealed that cyclin E1 overexpression significantly correlated with undifferentiated high-grade tumors (P < 0.001). Of the 138 tumors initially analyzed for cyclin E1 level, 94 tumors were reanalyzed focusing on detection of LMW derivatives. Western blot analysis of these extracts revealed that 15 (16%) exhibited elevated levels of bands that migrated between 25 to 49 kDa (Fig. 1A and B), corresponding to LMW forms of cyclin E1 described previously (10–13, 22). Statistical analysis indicated that the appearance of elevated levels of LMW derivatives was significantly associated with less-differentiated high-grade tumors as described previously (10). LMW derivatives were found associated with tumors of high-grade (P < 0.005) and negative hormone receptor status (estrogen, progesterone, and estrogen or progesterone; P < 0.005). However, in contrast to a previous study (10), Kaplan-Meier and Cox regression univariate analysis showed a trend between elevated levels of LMW derivatives of cyclin E1 or full-length cyclin E1 and overall or disease-free survival only for the cohort expressing very high levels of cyclin E1 and LMW derivatives (Fig. 2; Table 1). However, borderline statistical significance (P = 0.05) was only achieved for the correlation between...
very high levels of LMW cyclin E1 derivatives and disease-free survival and not for the other correlations (Table 1), most likely due to the small numbers in the respective “very high” cohorts. In a multivariate analysis, including cyclin E1, stage, grade, lymph node status, menopausal status, hormone receptor status, antiendocrine therapy, and chemotherapy, very high versus high/intermediate/low levels of cyclin E1 were independently associated with poor overall (hazard ratio (HR), 6.3; 95% confidence interval (95% CI), 1.8-22.4; \( P = 0.005 \)) and relapse-free survival (HR, 4.2; 95% CI, 1.2-14.5; \( P = 0.023 \)). Although the sample size for multivariate analysis, including LMW cyclin E1 derivative levels, was limiting, very high levels versus absent/low/high levels of LMW cyclin E1 derivatives were associated with relapse-free survival (HR, 22.1; 95% CI, 1.8-272.9; \( P = 0.016 \)). The sample size was too small to generate meaningful data for overall survival.

**LMW derivatives correlate with cyclin E1 levels in primary breast tumors and derived cell lines.** Western blot analysis revealed that LMW derivatives of cyclin E1 were detected almost exclusively in primary breast tumors that also expressed an elevated level of full-length cyclin E1 protein (Fig. 1). In fact, discordance between expression of LMW derivatives and elevated cyclin E1 was observed in only 1 of 94 tumors analyzed (data not shown). Statistical analysis revealed a highly significant correlation between elevated full-length cyclin E1 protein levels in extracts and detection of LMW derivatives in primary breast tumors (\( P < 0.001 \), \( \chi^2 \)).

Extended exposure of the Western blots (e.g., Fig. 1B) revealed that LMW derivatives of cyclin E1 can be detected in most tumor samples with adequate exposure to film. To further investigate the relationship between detection of LMW derivatives of cyclin E1 and cyclin E1 protein level, we reanalyzed a battery of breast tumor-derived extracts containing a range of cyclin E1 protein levels by Western blot analysis (two exposures shown in Fig. 3A). Although when sample loading was normalized to total cellular protein, LMW derivatives were detectable preferentially in extracts containing high levels of cyclin E1 (Fig. 3A); once loading was normalized to cyclin E1 levels, LMW derivatives were equivalently detectable (Fig. 3B). We further investigated the relationship between LMW derivatives of cyclin E1 and cyclin E1 expression levels in breast tumor-derived cell lines (Fig. 4). Cell lines used in

---

**Figure 2.** Kaplan-Meier analysis of overall and disease-free survival for patients from which tumor samples were obtained. A, cyclin E1, overall survival. B, cyclin E1, disease-free survival. C, LMW cyclin E1, overall survival. D, LMW cyclin E1, disease-free survival.
our analysis included (a) T-47D, which expresses a low level of full-length cyclin E1 protein, (b) MDA-MB-468, which expresses a moderately elevated level of cyclin E1 protein, and (c) MDA-MB-157, which expresses an extremely high level of cyclin E1 due to amplification of the CCNE1 gene locus (12). Western blot analysis of extracts normalized to total cellular protein detected a high level of LMW derivatives in extracts prepared from MDA-MB-157 cells, a moderate level of LMW derivatives in extracts prepared from MDA-MB-468 cells, and no LMW derivatives in extracts prepared from T-47D cells, in accordance with cyclin E1 expression levels (Fig. 4A). Western blots rerun with extract normalized for full-length cyclin E1 protein revealed that equivalent levels of LMW forms were detectable in all three extracts (Fig. 4A). Therefore, the presentation of LMW derivatives of cyclin E1 on Western blots is likely a detection issue directly related to the level of cyclin E1 protein and the duration of exposure of a developed blot to film.

Finally, we transfected the breast tumor-derived cell line MDA-MB-468 with varying amounts of a plasmid that expresses full-length FLAG-tagged cyclin E1 cDNA. As would be expected if detection of LMW derivatives was a function of total cyclin E1 levels, a coordinate increase in LMW derivatives of cyclin E1 was observed with increasing plasmid transfected (Fig. 4B). Furthermore, the banding pattern of the LMW derivatives of transfected cyclin E1 was identical to that observed for derivatives of endogenous cyclin E1, suggesting that they were generated by an analogous proteolytic cleavage. Collectively, these results show that the detection of LMW derivatives of cyclin E1 is a function of cyclin E1 protein levels.

**Generation of LMW derivatives of cyclin E1 is not tumor specific.** The exclusive detection of LMW derivatives of cyclin E1 in tumor or tumor-derived cells has led to the hypothesis that the proteolytic machinery responsible for their generation is tumor specific (22, 27). To test this hypothesis, we compared two samples of normal human breast tissue with samples from several different breast tumors (Fig. 5A). For the tumor samples, detection of LMW derivatives was proportional to the amount of protein loaded per gel lane. However, when samples were normalized to full-length cyclin E1, LMW derivatives from tumor samples could be detected in all lanes, although the relative ratios of the individual bands varied significantly from sample to sample (Fig. 5A, bottom). Surprisingly, similar results were obtained from the analysis of normal breast tissue, with banding patterns within the range of those observed in tumor samples (Fig. 5A, bottom, lanes 1 and 2). These data suggest that processing of LMW derivatives from full-length cyclin E1 protein is not a tumor-specific property. To confirm this conclusion, we increased the input levels of either tumor extracts or extract from normal breast tissue and found that LMW derivatives of cyclin E1 were progressively revealed (Fig. 5B).

Finally, we transfected nontransformed human telomerase (hTERT)-immortalized breast epithelial cells (33) with increasing concentrations of a cyclin E1 expression plasmid and analyzed the level of LMW derivatives generated. Expression of full-length cyclin E1 resulted in the efficient detection of LMW derivatives of cyclin E1 (Fig. 5C). Indeed, the relative level of LMW derivatives of cyclin E1 generated to full-length cyclin E1 expressed was equivalent in hTERT-immortalized breast epithelial cells compared with the breast cancer-derived cell line MDA-MB-468 (Fig. 4). These results indicate that the generation of LMW derivatives of cyclin E1 is not exclusively a tumor-specific property. However, due to the low numbers of normal breast tissue samples in our analysis, we cannot state that the typical banding patterns in normal breast tissue are absolutely identical to those observed in breast tumors.

**Table 1. Statistical analysis of breast cancer survival**

<table>
<thead>
<tr>
<th></th>
<th>Disease-free survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td>0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Grade</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Stage</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Cyclin E1 (very high)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LMW cyclin E1 (very high)</td>
<td>0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

Note: Univariate survival analysis (log-rank test). Data were obtained from an analysis of 138 primary breast tumors, including all analyzed for cyclin E1 protein level and 94 for LMW forms of cyclin E1. Abbreviation: NS, not significant.

**Figure 3.** LMW derivatives of cyclin E1 correlate with gene expression in primary tumors. A. Western blot analysis of cyclin E1 in eight primary breast tumors normalized to total cellular protein. Left, short exposure to detect full-length cyclin E1; right, longer exposure to detect LMW forms. B, Western blot analysis of the same tumors in (A) normalized for full-length cyclin E1. Note the roughly equivalent level of LMW forms in all extracts.
Discussion

Our data clearly show that the detection of LMW derivatives of cyclin E1 in breast tumors is primarily a function of the amount of cyclin E1 loaded per gel lane and not a variable linked independently to a property of the tumor itself. This was shown through (a) the highly statistically significant association between elevated cyclin E1 expression and detection of LMW derivatives in primary breast tumor extracts analyzed by Western blot (b) the relatively equivalent level of LMW derivatives detected in extracts from breast tumor-derived cell lines and breast tumors normalized for cyclin E1 protein, and (c) the coordinate increase in LMW derivatives in breast tumor-derived cell lines transfected with increasing amounts of cyclin E1-expressing plasmids leading to increased levels of cyclin E1 per gel lane while holding other variables constant. These results contradict a previous study that found cyclin E1 overexpression and LMW derivatives of cyclin E1 are independent factors correlated with poor outcome in patients with breast cancer (10).

The possible reason for the discrepancy between this current study and previous studies claiming that LMW derivatives of cyclin E1 are independent of cyclin E1 protein level could be due to differences in the technical procedures used in tumor extract preparation. We have found that the level of LMW derivatives of cyclin E1 in tumor extracts is influenced slightly by the lysis buffer and technical procedures used in extract preparation (data not shown). However, these modest differences cannot account for the significant discrepancy between the two data sets. On the other hand, the treatment of tumor samples postoperatively but before extract preparation may have a major effect on the integrity of cyclin E1, a variable we have not explored and may account for the dramatic differences in cyclin E1 degradation and concomitant appearance of LMW derivatives observed in other studies (10, 21). Nevertheless, taken together, our results suggest that the generation of LMW derivatives of cyclin E1 is likely to reflect a proteolysis artifact that arises after tumor resection and possibly during extract preparation. However, this proteolytic processing of cyclin E1 seems to occur equivalently in most, if not all, tumor samples, as evidenced when extracts are normalized to cyclin E1 levels. Indeed, normalization to cyclin E1 levels is critical in this type of analysis due to the intrinsic nonlinearity of detection methods used (e.g., X-ray film), which may create an illusion of differences in the ratios of full-length to LMW derivatives of cyclin E1 that do not exist.

Figure 4. Level of LMW derivatives of cyclin E1 in breast tumor-derived cell lines is dependent on expression. A, left, Western blot analysis of breast tumor-derived cell lines. Extract (50 μg) from asynchronously growing T-47D, MDA-MB-157, and MDA-MB-436 cells was separated on 10% SDS-PAGE gels and probed with anti-cyclin E1 antibody HE12. Right, Western blot of breast tumor-derived cell lines normalized for full-length cyclin E1 protein. Extracts were serially diluted, and the level of full-length cyclin E1 protein was initially compared by Western blot analysis. Western blots were then rerun normalized for full-length cyclin E1. B, MDA-MB-468 cells were transfected with increasing amounts (2, 4, 8, and 16 μg) of expression plasmid pFlag-CMV2-cyclin E1. After 48 hours, the presence of LMW derivatives of cyclin E1 was detected by Western blot analysis using antibody HE12. C, nontransfected control.

Figure 5. LMW derivatives of cyclin E1 are not tumor specific. A, Western blots of two normal breast tissue samples (N) and four breast tumor samples (T). Top, Western blot for cyclin E1 normalized to total protein; middle, γ-tubulin loading control for top; bottom, Western blot for cyclin E1 normalized to total protein. B, Western blot analysis of LMW forms of cyclin E1 in normal breast tissue and primary breast tumors. Increasing amounts (12.5, 25, or 50 μg) of extract from two primary breast tumor specimens (T1 and T2) and a normal breast mammoplasty (N) were analyzed. C, hTERT-immortalized breast epithelial cells were transfected with increasing amounts of pFlag-CMV2-cyclin E1 as described.
Of equal concern with regard to using detection of LMW derivatives of cyclin E1 as a marker for prognostic purposes or the idea of therapeutic approaches targeting the putative protease itself, our data indicate that the presentation of LMW derivatives of cyclin E1 in extracts is not exclusive to malignancy. Extracts from normal breast tissue and hTERT-immortalized nontumored human mammary epithelial cells were found to exhibit LMW derivatives when cyclin E1 levels were increased by ectopic expression or when increasing amounts of extract were loaded. In fact, the level of LMW derivatives generated relative to full-length cyclin E1 in immortalized mammary epithelial cells was comparable with that observed for extracts prepared from the breast cancer-derived cell line MDA-MB-468. These data suggest that the protease that cleaves full-length cyclin E1 protein into LMW derivatives is active in nontumor-derived cells that exhibit no markers of malignancy. The pattern of LMW derivatives generated in immortalized mammary epithelial cells was identical to that observed in primary tumor extracts and transfected MDA-MB-468 cells, suggesting that the LMW derivatives were generated through the same molecular mechanism.

In a previous study, it was reported that both total cyclin E1 level and level of LMW derivatives showed a strong inverse correlation with overall and disease-free survival (10). Our data differ somewhat in that we only observed such a correlation in the cohort of patients with tumors expressing very high levels of cyclin E1, this being a relatively small subpopulation (5.8%), and the trend observed did not reach significance with the sample size under investigation. Tumors with very high levels of LMW derivatives (5.3%) also were a predictor of reduced survival. However, because the tumors with very high levels of cyclin E1 were, for the most part, the same as those exhibiting high levels of LMW derivatives, these were not independent prognostic markers in our study. On the other hand, patients with tumors that expressed high but not very high levels of cyclin E1 did not show decreased survival. The discrepancies between the two studies might be explained if the cutoff levels for cyclin E1 scoring were set differently. However, this is not consistent with the relative sizes of the different cohorts. It is also possible that patients in the two studies were subjected to different therapeutic regimes affecting patient survival. In addition, the ethnicity of the patients may be a factor, as the patient samples in the previous study were obtained in the United States, whereas those in our study were of Austrian origin. Additional research will be required to resolve this discrepancy.

Acknowledgments
Received 9/8/2005; revised 4/14/2006; accepted 5/5/2006.
Grant support: NIH grant CA78343 (S.I. Reed).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank J. Shay for hTERT-immortalized human mammary epithelial cells.

References
Detection of Low Molecular Weight Derivatives of Cyclin E1 Is a Function of Cyclin E1 Protein Levels in Breast Cancer

Charles Spruck, Dahui Sun, Heidi Fiegl, et al.


**Updated version**
Access the most recent version of this article at:
[http://cancerres.aacrjournals.org/content/66/14/7355](http://cancerres.aacrjournals.org/content/66/14/7355)

**Cited articles**
This article cites 32 articles, 14 of which you can access for free at:
[http://cancerres.aacrjournals.org/content/66/14/7355.full.html#ref-list-1](http://cancerres.aacrjournals.org/content/66/14/7355.full.html#ref-list-1)

**Citing articles**
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
[http://cancerres.aacrjournals.org/content/66/14/7355.full.html#related-urls](http://cancerres.aacrjournals.org/content/66/14/7355.full.html#related-urls)

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.