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Cyclin E deregulation is an early event in the development of breast cancer

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Abstract

Cyclin E has been shown to be overexpressed in some human breast cancers, however, data to support deregulation of cyclin E as an early event in human mammary tumor development is lacking. We analyzed surgical specimens from 183 patients with breast carcinomas and evaluated cyclin E expression in areas of invasive carcinoma, adjacent carcinoma in situ (CIS), and non-neoplastic breast parenchyma. Overexpression of cyclin E was seen in one-third of invasive carcinoma samples, one-third of the CIS component and nearly half of the non-neoplastic breast epithelial cells adjacent to carcinoma (44% vs. 33%, $P = 0.05$). Nuclear labeling for cyclin E was highly concordant between areas of invasive carcinoma, CIS and non-neoplastic breast epithelial cells from the same patient ($P < 0.0001$). Localization of cyclin E to the cytoplasm was seen in a small proportion of tumor samples. Our findings suggest that cyclin E deregulation is an early event in the progression from histologically benign mammary epithelial cells to invasive carcinoma and occurs through both overexpression and altered cellular localization.

Keywords

Cyclin E; Premalignant breast; disease Breast cancer; Cytoplasmic localization

Introduction

Cell cycle deregulation is a hallmark of cancer. The G1/S checkpoint in particular appears to be the most relevant to the process of carcinogenesis and is invariably deregulated in human tumors [1]. Cyclin E, together with its kinase partner cdk2, is a key mediator of the transition from G1 to S phase [2], regulating the forward momentum through G1 into S phase. Therefore, alterations of cyclin E will result in profound effects on the G1/S transition with overexpression of cyclin E favoring rapid transit through G1 and resistance to exit from the cell cycle. In support of the important role of cyclin E as a gatekeeper of the G1/S transition, cyclin E overexpression has been reported in a number of malignancies including breast, ovarian, gastric, non-small cell lung and adrenocortical carcinomas as well as non-Hodgkin's lymphoma and in almost all cases has been shown to correlate with a more aggressive tumor phenotype and adverse prognosis [3–12].

Cyclin E deregulation has been most extensively studied in breast cancer where it has been shown to be deregulated in approximately 25% of patients with invasive carcinoma [7]. Alterations of cyclin E are seen at the level of gene amplification, overexpression of mRNA as well as posttranslational modification of the full-length (FL) cyclin E into several low molecular weight forms (LMW) [13].

Several lines of evidence suggest that alteration of cyclin E is oncogenic in breast cancer. In mammalian cell systems, transfection of the LMW isoforms into normal mammary epithelial cells has significant mitogenic effects, readily inducing cells to enter the cell cycle [14]. Deregulation of cyclin E has been linked to chromosomal instability, mammary tumor development in transgenic mouse models and resistance to anti-estrogens [15–17]. Recently, cyclin E has also been shown to activate DNA damage signaling [18, 19]. Cyclin E is a strong prognostic marker in breast cancer with those patients having cyclin E overexpression in the primary tumor having a poorer overall and disease-free survival when compared with patients whose tumors do not overexpress cyclin E [7, 8].

Although cyclin E has been studied in invasive breast carcinoma, there is no information about its expression and biologic effects in pre-malignant lesions such as ductal carcinoma in situ or in high risk, but non-malignant lesions such as atypical ductal hyperplasia. Two groups have reported that cyclin E is overexpressed in a small cohort (5–25%) of ductal carcinoma in situ (DCIS) patients [20, 21], however, cyclin E expression in pre-malignant lesions has not been correlated with expression in corresponding areas of invasive carcinoma. Such a link is necessary to demonstrate that cyclin E mediates early changes in breast mammary epithelial cells with resultant step-wise progression to pre-malignant and finally malignant breast disease. Therefore, in human breast cancer, a clear linear progression of cyclin E driven oncogenesis from premalignant to malignant disease has yet to be established. The goal of this study was to correlate cyclin E expression in primary breast cancer (stage I and II) with expression of cyclin E in associated areas of in situ

carcinoma in order to determine whether cyclin E may be involved in the progression from pre-malignant to invasive disease.

Materials and methods

Patient samples

Tumor and normal tissue samples for this study were obtained from patients enrolled in an IRB approved protocol to prospectively study the prognostic impact of cyclin E deregulation in patients with breast cancer. Although the study inclusion criteria included all patients with operable breast cancer, for purposes of the current analysis, only patients with stage I or II breast cancer who did not receive preoperative chemotherapy were included.

Immunohistochemistry

A representative section of invasive tumor and in situ carcinoma was selected and stained with the polyclonal C-19 antibody to cyclin E (Santa Cruz) at a concentration of 1:300. Since there is no standardized antibody for cyclin E analysis in human tissue samples, we first tested the C-19 antibody on a test set of replicate samples at concentrations ranging from 1:300 to 1:800 in order to identify the staining that optimized sensitivity with minimal background staining (high specificity). Staining intensity and percent positivity were evaluated both in the nucleus and cytoplasm in invasive and in situ carcinoma as well as in non-neoplastic breast epithelial cells by a single pathologist. Similarly there is no standardized cut-off to determine cyclin E positivity. Most reports describe cut-points between 5 and 10% nuclear staining [21–25] although a few have reported cyclin E positivity with frequencies as low as 2% [26] and some as high as 50% [27]. Most publications rely on percent staining, with few combining both percent staining with intensity [3, 28]. We chose a combined approach to more stringently identify cases with cyclin E overexpression. Thus, samples were considered to overexpress cyclin E when >5% of cells showed >1+ nuclear staining intensity. Cytoplasmic staining of cyclin E was scored as positive if homogenous staining was noted throughout the cytoplasm. Correlation between cyclin E overexpression in invasive, in situ and non-neoplastic epithelial cells in sections obtained from each patient was determined. Staining with ser-139 phospho-H2AX was performed using a monoclonal antibody (Upstate) at a dilution of 1:500. Samples were scored positive if >5% of cells showed staining.

Cell lines and culture conditions

MCF-7 cell lines overexpressing cyclin E were generated as previously described [17]. Empty vector and cyclin E overexpressing clones were maintained in α -MEM with 10% FBS and selection of the transgene maintained with 80 μ g/ml of zeocin. All cells were cultured and treated at 37C in a humidified incubator containing 6.5% CO₂.

Western blot assay

For western blot analysis, cells were homogenized by sonication and high speed centrifugation. The cleared cell lysate supernatant was assayed for total protein content and then subjected to western blot analysis as previously described [29]. Briefly, 25–50 μ g of protein from each condition was subjected to electrophoresis in each lane of a 10% (cyclin

E, tubulin) and 7% (PARP-1) sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to Immobilon P for 2 h at 4°C at 85 mV constant volts. The blots were blocked overnight at 4°C in Blotto (5% nonfat dry milk in 20 mM Tris, 137 mM NaCl, 0.25% Tween, pH 7.6). After 6, 10 min washes in TBST (20 mM Tris, 137 mM NaCl, 0.05% Tween, pH 7.6), the blots were incubated in primary antibodies for 2 h. Primary antibodies used were cyclin E C-19, PARP-1 (H-250) (both from Santa Cruz Biochemicals, Santa Cruz, CA), and tubulin (TUB 2.1, Sigma St Louis MO) all at 1 µg/ml in Blotto. Following primary antibody incubation, the blots were washed and incubated with goat anti-mouse or goat antirabbit horseradish peroxidase conjugate at a dilution of 1:5000 in Blotto for 1 h and finally washed and developed with the Renaissance chemiluminescence system as directed by the manufacturers (NEN Life Sciences Products, Boston, MA).

Nuclear and cytoplasmic fractionation

The nuclear and cytoplasmic extracts from breast cancer cell lines were isolated using a NE-PER[®] Nuclear and Cytoplasmic Extraction Kit from PIERCE (Rockford, IL). Approximately 20 µl packed cell volume (~40 mg) of cells were collected by centrifugation at 500×*g* for 5 min. Two hundred microliters of ice-cold CER I (from the kit) were added into the cell pellet, the tubes were vigorously vortexed for 15 s to fully re-suspend the cell pellet. The mixture was incubated on ice for 10 min and then 11 µl of ice-cold CER II (from the kit) was added into the mixture. The mixture was vortexed and incubated on ice for 1 min, then centrifuged for 5 min at 16,000×*g*. After the centrifugation the supernatant (cytoplasmic extract) fraction was immediately transferred to a clean pre-chilled tube. The pellet was washed with sterilized 1 × PBS 3 times, and re-suspended in 100 µl of ice-cold NER (from the kit). The re-suspended pellet was vortexed for 15 s, and incubated on ice for 10 min. The vortex/incubation process was repeated for 4 times. After the final vortex step, the samples were centrifuged at 16,000×*g* for 10 min. The supernatant (nuclear extract) fraction was immediately transferred to a clean pre-chilled tube. Both extracts were stored at -80°C until analysis.

Statistical analysis

Fisher's exact test was utilized to assess the association between cyclin E overexpression and the factors of interest. In order to test for the concordance of cyclin E expression between the various compartments (non-neoplastic epithelial cells, in situ carcinoma and invasive carcinoma) and between cytoplasmic and nuclear compartments, data were analyzed using an exact binomial test to assess whether the proportion of observations that were concordant [(+,+) or (-,-)] was significantly greater than 50%. Ninety-five percent confidence intervals around the proportions of concordant observations are provided as well. Statistical significance was confirmed if $P \leq 0.05$.

Results

Cyclin E expression in breast tumor tissue

One hundred and eighty-three samples from patients with stage I and stage II breast cancer were available for analysis. Sections from 55 cases demonstrated no in situ disease and conversely, in 6 cases, the available sections showed no invasive component. Thus a total of

177 invasive cancer and 128 in situ carcinoma cases were available for cyclin E analysis. Additionally, in 166 samples, areas of nonneoplastic breast epithelium was evident adjacent to the tumor; these cases formed the basis of cyclin E expression in non-neoplastic breast epithelium.

Figure 1 demonstrates representative slides of cyclin E overexpression in non-neoplastic breast epithelium, in situ carcinoma and invasive carcinoma. Overall, cyclin E overexpression was evident in 1/3 (58/177 patients, 33%) of invasive cancers and similarly 1/3 (42/128 patients, 33%) of in situ cases. In contrast, in 73/166 patients (44%), non-neoplastic breast epithelial cells showed overexpression of cyclin E protein (Fig. 2). This higher proportion of cyclin E deregulation in non-neoplastic breast epithelial cells compared to in situ carcinomas and invasive carcinomas was statistically significant ($P=0.05$ and 0.03 , respectively). Relative to the invasive cancer component, sensitivity and specificity of cyclin E staining in non-neoplastic mammary epithelium and in situ cases were 67%, 68%, 66% and 85%, respectively.

Concordance of cyclin E expression between non-neoplastic breast epithelium, in situ and invasive carcinoma

We next examined whether cyclin E overexpression was concordant between each of the three types of tissue compartments in each individual patient: non-neoplastic breast epithelium, in situ carcinoma and invasive carcinoma. For this analysis, areas were considered concordant if both overexpressed cyclin E or neither overexpressed the protein. As can be seen in Table 1, in the majority of cases (95/122, 78%), nuclear labeling for cyclin E in invasive carcinoma was accompanied by similar expression levels in associated areas of CIS. Similarly, cyclin E expression in non-neoplastic breast epithelial cells was highly predictive of cyclin E expression in adjacent areas of in situ carcinoma (79/120, 66%) and invasive carcinoma (108/160, 68%). Overall, cyclin E overexpression was highly concordant between non-neoplastic breast epithelial cells and areas of in situ carcinoma and invasive carcinoma as well as between areas of in situ carcinoma and invasive carcinoma ($P<0.0001$ for all comparisons). In patient samples where cyclin E overexpression was noted in non-neoplastic breast epithelial cells, 61% (45/73 cases) had overexpression of cyclin E in areas of CIS and/or invasive carcinoma. Importantly, in non-neoplastic breast epithelial cells without cyclin E staining, 77% (71/93 cases) of cases also had negative staining in associated areas of CIS/invasive carcinoma ($P=0.0001$). Only nine cases showed cyclin E overexpression in the normal mammary epithelium and associated invasive cancer without corresponding overexpression in areas of in situ carcinoma.

To provide further support that cyclin E deregulation we noted in histologically normal breast epithelial cells in these breast cancer samples is biologically significant and not staining artifact, we evaluated for the presence of DNA damage by staining for γ -H2AX. Multiple oncogenes, including cyclin E have been shown to activate DNA damage [19] suggesting a common pathway that is upregulated largely independent of the original oncogenic stimulus. We found that DNA damage is present in 77%, 72% and 66% of invasive cancer, in situ cancer and nonneoplastic tissues, respectively. As shown in Fig. 3, we found γ -H2AX staining to be equally strong in histologically normal mammary

epithelium and adjacent invasive cancer cells. Additionally, we found that among the 73 cases with cyclin E overexpression in non-neoplastic cells, in the majority (50 cases, 68%), histologically normal breast epithelial tissue samples also stained positive for ser-139 phospho-H2AX. Our finding that DNA damage is present in the majority of the non-neoplastic cells we examined would suggest that specific oncogenic abnormalities in these cells, such as cyclin E overexpression, represent a biologically valid event rather than non-specific staining and thus non-significant finding. Our data thus supports the hypothesis that cyclin E deregulation occurs early in the process of malignant transformation, occurring even before histological changes of malignancy are evident.

Altered localization of cyclin E in human mammary tumors

We also found that cyclin E deregulation occurred through altered localization of the protein (Fig. 1d showing cytoplasmic expression compared to Fig. 1f with nuclear expression only). Cytoplasmic expression of cyclin E was noted in 13% (21/166 cases), 24% (31/128 cases) and 14% (25/177 cases) of non-neoplastic breast epithelial cells, in situ carcinoma and invasive carcinoma cells, respectively. Within in situ carcinomas and invasive carcinoma cells, cyclin E deregulation at the level of altered cellular localization strongly correlated with cyclin E overexpression in the nucleus ($P < 0.0001$, Table 2).

In order to extend these observations that cyclin E deregulation also appears to occur through altered cellular localization of the protein, we next examined several breast cancer cell lines for localization of cyclin E. Cells were fractionated and nuclear and cytoplasmic extracts tested for cyclin E expression. Tubulin and PARP-1 expression in these extracts were also examined to confirm appropriate cytoplasmic and nuclear fractionation, respectively. As can be seen in Fig. 4, all the tumor cell lines showed altered localization of cyclin E with expression of the protein in both the nucleus and the cytoplasm. Expression levels of cyclin E appeared to be similar between the nuclear and cytoplasmic compartments. Additionally, even in breast tumor cell lines with normal levels of cyclin E expression (i.e., T47D), cytoplasmic expression was evident. This in vitro data confirms our findings in patient samples and underscores that cyclin E deregulation occurs through more than just overexpression of the protein.

Association between cyclin E and other histopathologic variables

Cyclin E overexpression has been reported to be associated with more aggressive tumor phenotypes such as higher grade and low estrogen receptor status [22, 30, 31]. We similarly examined these variables in our series. For this analysis, we limited the analysis to cyclin E expression in the areas of invasive cancer and compared to ER, her-2/neu, grade and ki-67 expression again in the invasive component of the tumor. We found no statistically significant associations between cyclin E overexpression and ER status, her2/neu expression, grade or proliferation (as determined by ki-67 labeling). However, when we examined triple negative tumors compared to all other combinations of ER, PR and her-2/neu, we found cyclin E overexpressing tumors to be significantly more likely to carry a triple negative phenotype (67% vs. 33%, $P = 0.021$) (Table 3).

Discussion

Cyclin E as an oncogene in breast cancer has been suggested by the biological role of the protein [2, 15, 17, 32, 33], reports of cyclin E overexpression in human breast cancers [7, 27, 28, 34] and the development of breast tumors in cyclin E mouse transgenic models [16, 35]. Our current findings further strengthen the link between cyclin E and breast cancer by demonstrating that cyclin E deregulation occurs early in the oncogenic process and is present prior to the development of in situ carcinoma and subsequent progression to invasive carcinoma. Furthermore, our report makes the novel observation that cyclin E is deregulated in tumors not just at the level of protein expression but also through altered cellular localization.

Our results show that nuclear cyclin E is overexpressed in approximately one-third of in situ carcinomas of the breast. This is comparable to data from Jirstrom et al. [20], who estimated that 25% of patients with DCIS will have overexpression of cyclin E in the tumor cells but is significantly greater than the 5% staining reported in a small series by Scott et al. [21]. The larger cohort size in our study and in the Jirstrom study as well as differences in staining techniques may account for differences in frequency of cyclin E deregulation in DCIS. Importantly, in our study we compared cyclin E expression between in situ carcinomas and invasive carcinomas. We found that levels of cyclin E are similar in invasive and in situ carcinoma (33% show overexpression of cyclin E) and that there is very high concordance between cyclin E expression in the in situ and invasive components of the tumor. This finding provides evidence that cyclin E overexpression is initiated prior to the development of invasion.

In addition to showing high concordance of cyclin E overexpression between in situ and invasive disease, we were able to extend the observation further, demonstrating that cyclin E deregulation can also be observed within nonneoplastic breast epithelial cells adjacent to the tumor mass. A prior study investigating cyclin E overexpression in normal breast tissue showed no reactivity in non-neoplastic breast epithelial cells [21]. Although this is in contrast to our findings, there is a critical distinction between the 2 studies. Whereas Scott and colleagues looked at normal cells at least 4 cm away from the tumor mass, the non-neoplastic epithelial cells in our study are directly adjacent to areas of carcinoma. A number of observations suggest the relevance of these findings to the early stages of breast tumorigenesis. First, we expressly set a high threshold for determining cyclin E overexpression and included only samples with high expression level by both percent positivity *and* staining intensity. Second, we find a strong association between cyclin E overexpression in non-neoplastic breast epithelial cells and in associated areas of pre-malignant and malignant disease. Third, in samples where cyclin E overexpression was not noted in normal epithelial cells, nearly 80% of adjacent in situ and invasive cells similarly lacked cyclin E overexpression. And lastly, a high proportion of histologically normal cells also showed evidence of DNA damage, suggesting that molecular abnormalities such as cyclin E overexpression in non-neoplastic cells are biologically real observations.

There are a number of implications to our finding that cyclin E is overexpressed in adjacent non-neoplastic breast epithelial cells. First, it demonstrates that there is a molecular field

defect in breast cancer that extends beyond the border of the areas of histological abnormality. Whether this molecular field defect has clinical significance, such as increasing the probability of an adverse local event requires further evaluation. Second, it strengthens the association between cyclin E and breast tumorigenesis. Lastly, the relatively higher expression of cyclin E in nonneoplastic areas compared to areas of carcinoma, suggest that cyclin E may an important initiator of the tumorigenic process leading to cellular transformation and setting into motion subsequent aberrations, such as genomic instability, which then drives progression to carcinoma, even if cyclin E overexpression is lost. Further proof that cyclin E may be required for initiation, but not maintenance of the tumors comes from cyclin E transgenic mice, where approximately 25% of mammary carcinomas lose expression of cyclin E despite retention of the transgene in contralateral unaffected mammary gland [35].

The biology of cyclin E overexpression is largely attributed to the function of cyclin E, in association with cdk2, within the nucleus. Our results showing cyclin E cytoplasmic expression in tumor samples as well as breast tumor cell lines provide preliminary evidence that cyclin E may also be deregulated during tumorigenesis through altered cellular localization. Of interest, there was a strong association between cyclin E overexpression in the nucleus and its expression in the cytoplasm of in situ and invasive carcinomas, but not in non-neoplastic breast epithelial cells. This suggests that the earliest stages of tumor development, cyclin E deregulation can occur either through overexpression of protein or altered localization to the cytoplasm. However, in more advanced stages of tumor genesis, both forms of deregulation are likely to be present. Of interest, recent data show that cyclin E may drive cell cycle progression in a kinase independent fashion [36]. These data challenge the conventional notion of cyclin E activity limited to its association with cdk2. Similarly, our findings open the possibility that the presumed nuclearlimited function of cyclin E may also under-represent the full scope of cyclin E's biologic functions.

Cyclin E has been shown to shuttle between nucleus and cytoplasm [37]. However, how cyclin E is transported into and out of the nucleus remains controversial. Additionally, the functional role of cyclin E overexpression in the cytoplasm and the potential implications for tumorigenesis remain unexplored. One of the substrates of cyclin E/cdk2 is nucleophosmin B23, which when phosphorylated dissociates from the centrosome, opening the way for centrosomal duplication [38]. In *Xenopus*, increased levels of cyclin E/cdk2 in the cytoplasm during interphase leads to accelerated entry into mitosis [39] although these findings were not reproduced in Hela cells microinjected with cyclin E/cdk2 during G2 [40]. Nonetheless, these observations open up the possibility that overexpression of cyclin E in the cytoplasm may deregulate cell cycle kinetics and enhance centrosome duplication, both of which could contribute to the overall oncogenic properties of cyclin E.

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Fig. 1.

Representative sections from breast tumors with different cyclin E staining patterns. **a** H and E normal breast parenchyma; **b** cyclin E immunostaining showing nuclear staining in normal breast ductal cells; **c** H and E in area of DCIS; **d** cyclin E immunostaining of area of DCIS showing nuclear staining and in some areas cytoplasmic staining (arrows); **e** H and E in area of invasive carcinoma; **f** cyclin E immunostaining showing nuclear staining only in invasive carcinoma cells; **g** cyclin E immunostaining in invasive carcinoma cells (arrows) and

adjacent normal breast ducts (*arrowheads*); **h** negative control sample stained with secondary antibody only; no background staining is noted. All images shown at $\times 20$ magnification

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Fig. 2.

Cyclin E overexpression in breast cancer specimens. Cyclin E expression is significantly higher in non-neoplastic breast epithelial cells and decreases to approximately one-third of cases showing nuclear overexpression of cyclin E in in situ and invasive carcinomas (* $P=0.05$ relative to non-neoplastic cells; ** $P=0.03$ relative to non-neoplastic breast epithelial cells)

Fig. 3. Breast tumors stained for ser-139 phosphorylated histone H2AX shows presence of γ -H2AX foci in both ductal carcinoma in situ (DCIS) (*arrows*) and adjacent normal breast ducts (*arrowhead*) (**a**), Similarly strong expression is seen in invasive breast carcinoma (**b**)

Fig. 4. Altered localization of cyclin E is seen in breast tumor lines. Cell lines indicated were fractionated into nuclear and cytoplasmic fractions and then analyzed for expression of cyclin E by western blot assay. PARP and β -tubulin expression were used to indicate nuclear and cytoplasm fractions, respectively. Near equal expression of cyclin E is seen in both nuclear and cytoplasmic compartments

Table 1

Correlation between cyclin E overexpression in non-neoplastic breast epithelium, in situ carcinoma and invasive carcinoma compartments

Concordance	Proportion	Exact 95% CI	P-value
Nuclear concordance between in situ and invasive carcinoma	95/122 (78%)	0.69–0.85	<0.0001
Nuclear concordance between in situ carcinoma and histologically normal adjacent tissue	79/120 (66%)	0.57–0.74	<0.0001
Nuclear concordance between invasive carcinoma and histologically normal adjacent tissue	108/160 (68%)	0.60–0.75	<0.0001

Table 2.

Nuclear and cytoplasmic concordance for cyclin E deregulation

Concordance	Proportion	Exact 95% CI	P-value
Invasive carcinomas: nuclear and cytoplasmic staining	122/177 (69%)	0.62–0.76	<0.0001
In situ carcinomas: nuclear and cytoplasmic staining	85/128 (66%)	0.58–0.75	<0.0001
Non-neoplastic breast epithelium: nuclear and cytoplasmic staining	94/166 (57%)	0.49–0.64	0.10

Table 3.

Association between cyclin E and histopathologic variables *

	Cyclin E positive	Cyclin E negative	P-value
ER (<i>n</i> = 175)			
Positive	48 (31%)	105 (69%)	0.23
Negative	10 (45%)	12 (55%)	
Hormone status (ER/PR/Her-2/neu) (<i>n</i> = 170)			
Triple negative	8 (67%)	4 (33%)	0.021
Other	48 (30%)	110 (70%)	
Her-2/neu (<i>n</i> = 169)			
Positive	4 (20%)	16 (80%)	0.22
Negative	52 (35%)	97 (65%)	
Grade (<i>n</i> = 175)			
1/2	36 (29%)	89 (71%)	0.11
3	22 (42%)	30 (58%)	
Ki67 (<i>n</i> = 100)			
Low/mod	22 (29%)	55 (71%)	0.21
High	10 (43%)	13 (57%)	

*Histopathologic data not available in all cases