Prognostic Influence on Survival of Increased Ornithine Decarboxylase Activity in Human Breast Cancer

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ABSTRACT

Although considerable experimental evidence suggests an important role of polyamines in breast cancer biology, compelling supportive data in patients are lacking. To address this issue, we measured ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase, and spermidine/spermine acetyltransferase (the three key polyamine metabolic enzymes) in a cohort of 50 primary human breast cancers and related their levels of activity to disease-free survival and overall survival. The major finding of our study was that ODC activity level was a negative independent prognostic factor for both end points. With regard to overall survival, the adverse influence of ODC expression was superior even to that provided by the number of positive nodes. Furthermore, the statistical significance of the ODC effect on survival was enhanced when breast cancer-specific mortality was included in the analysis as opposed to death from any cause. In addition, high tumor ODC activity may predict a shorter time from recurrence to death, although this effect was of only borderline statistical significance. In summary, these results provide the first concrete evidence supporting the prognostic role of ODC in human breast cancer.

INTRODUCTION

Considerable evidence derived from a variety of in vitro and in vivo experimental systems indicates that polyamines (putrescine, spermidine, and spermine) are critically involved in breast cancer cell proliferation (1-4). Furthermore, we have recently provided evidence to suggest that activation of the polyamine biosynthetic pathway may be involved in breast cancer progression to a less hormone-responsive and more aggressive phenotype (5, 6).

To test the clinical relevance of our findings, we measured the activities of the three key polyamine metabolic enzymes (ODC,3 SAMDC, and SSAT; Fig. 1) in a cohort of 50 primary human breast cancers with known clinical follow-up. The major objective of our study was to determine whether the levels of enzymatic activity have any prognostic impact on disease-free survival and overall survival.

MATERIALS AND METHODS

Tumor Samples. Fifty primary breast cancer specimens were randomly chosen from a subset of the breast cancer prognostic study collection of the Karmanos Cancer Institute (Detroit, MI). This large tumor bank has been described in detail elsewhere (7, 8). Tumor specimens were sectioned and transported on ice to a central laboratory, where they were frozen at -70°C within 1 h of surgery. Specimens were selected from among those that were large enough to yield adequate tissue for the polyamine metabolic enzyme assays (i.e., ~1 g). Samples were coded at selection and not decoded until completion of the laboratory investigations. The demographic characteristics of the patients and tumor samples used in this investigation are summarized in Table 1. The mean duration of follow-up in this group of patients is 74 ± 58 (SD) months. Twenty-eight patients (56%) have recurred. All of them have died of breast cancer. An additional 13 patients (26%) have died of either unrelated (n = 9) or unknown (n = 4) causes. Nine patients (18%) are still alive and free of disease. Recurrence time and overall survival time were defined as the time from primary breast cancer diagnosis at surgery to the first recurrence and to death, respectively.

Biochemical Assays. Frozen specimens were homogenized in buffer containing 5 mM NH₄PO₄, 0.1 mM EDTA, and 2 mM dithiothreitol (pH 7.4). Separate aliquots of the homogenates were stored at -70°C. At the time of the assay, the samples were thawed, sonicated for 20 s, and centrifuged at 100,000 X g for 30 min. ODC, SAMDC, and SSAT activities were determined in the supernatants according to standard methodologies. ODC activity was measured according to the method described by Pegg et al. (9) with minor changes as published by us (6). Enzyme activity was calculated by measuring the picomoles of 14CO₂ liberated from [1-14C]-L-ornithine over a 30-min period under conditions of linearity of the assay with regard to time and protein concentration. ODC activity was expressed as pmol/mg protein/30 min. SAMDC activity was similarly assayed using [carboxyl-14C]S-adenosylmethionine as a substrate in the presence of saturating putrescine (3 mM) as described by Pegg and Poso (10). A detailed methodology for determination of SSAT activity has been recently published by us (6).

Statistical Analysis. The prognostic value of the three polyamine metabolic enzymes with respect to disease-free sur-

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3 The abbreviations used are: ODC, ornithine decarboxylase; SSAT, spermidine/spermine acetyltransferase; SAMDC, S-adenosylmethionine decarboxylase; ER, estrogen receptor; CI, confidence interval.
vival and overall survival was assessed by proportional hazards regression analysis (11). Each enzyme was evaluated individually by univariate analysis, and after adjusting for the demographic variables in a multivariate analysis. These included ER status, age, tumor size, the number of excised nodes, and the number of positive nodes. In addition, because disease recurrence is often a precursor to death, multivariate analysis of overall survival including disease recurrence as a time-dependent indicator variable (12) was also done. To meet model assumptions, the polyamine metabolic enzymes were analyzed after logarithmic transformation as continuous variables and as dichotomous variables (dichotomized at the median). All analyses were carried out using the SAS statistical software system (13).

RESULTS

Fig. 2 depicts the individual enzyme measurements in our patient population subdivided according to ER status. As can be seen, the levels of ODC, SAMDC, and SSAT were not significantly different in ER-positive and -negative tumors. In addition, the degree of expression of the three enzymes in the tumors was not significantly correlated with any of the demographic variables. ODC was moderately correlated with SAMDC on the log scale ($r = 0.43; P = 0.002$). SSAT was also moderately correlated with SAMDC on the log scale ($r = 0.34; P = 0.03$).

Univariate analysis revealed that ODC was a significant prognostic factor for both disease recurrence and death. Increased ODC activity is associated with an increased risk of both disease recurrence and death. Neither SSAT nor SAMDC was a significant factor for disease recurrence or death. Table 2 summarizes the results of the univariate survival analysis for the three enzymes on the log scale. Because the enzyme variables were analyzed on the log scale, the risk ratio can be interpreted as the relative risk associated with a 10-fold increase in enzyme activity. Similar results were obtained when the enzymes were analyzed as dichotomous variables. The prognostic significance of the three enzymes was also assessed with regard to disease-specific mortality in the group of 28 patients who died of breast cancer. Log (ODC) was found to be highly significant ($P = 0.008$), with a relative risk of 1.95 (95% CI, 1.19-3.20). Again, similar results were obtained when the enzymes were analyzed as dichotomous variables.

After adjusting for the other known variables, multivariate analysis revealed that both ODC and SSAT were significant prognostic factors for disease recurrence (Table 3). The number of positive nodes was the only other significant factor for disease recurrence. With respect to overall survival, ODC and the number of positive nodes were the only significant prognostic factors. It should be noted that the prognostic influence of ODC was superior to that provided by the number of positive nodes. ODC was also a significant predictor of disease-specific mortality, with log (ODC) showing a relative risk of 3.79 (95% CI, 1.43-10.07; $P = 0.008$). Neither SSAT nor SAMDC was a significant prognostic factor for death. The fact that SSAT was significant in the multivariate analysis of disease-free survival but not in the univariate analysis may be indicative of an interactive effect between SSAT and one of the other variables, although no significant interaction effects were found in our analysis.

Because ODC and the number of positive nodes were significant prognostic factors for both recurrence and death, this raises the question of whether the prognostic value of these variables for death was merely a reflection of their association with recurrence, which in turn would be associated with death. To address this issue, we introduced disease recurrence as a time-dependent covariate in the multivariate analysis of overall survival. Table 4 summarizes the results of this analysis. As anticipated, recurrence was found to be the strongest predictor of death, with a risk ratio of 13.8 ($P = 0.0001$). The number of positive nodes was not significant in this analysis, indicating that its prognostic value for overall survival was primarily due to its association with disease recurrence. ODC, on the other hand, maintained a prognostic influence that was of borderline statistical significance ($P = 0.08$). Fig. 3 depicts the effect of a 10-fold difference in ODC activity on overall survival in these patients. This finding suggests that a high ODC activity may
identify a subgroup of breast cancer patients at higher risk of death after relapse has occurred. To further address this issue, we analyzed the influence of ODC activity on time from recurrence to death in the group of 28 patients who relapsed and died of breast cancer. In this analysis, we found that the relative risk for log (ODC) was 1.74 (95% CI, 0.91–3.32; \( P = 0.09 \)). Essentially, the same results were obtained when ODC was analyzed as a dichotomous variable. These results support the hypothesis that high tumor ODC activity is associated with shorter time from recurrence to death, although the influence of ODC on this parameter was, again, of only borderline significance.

**DISCUSSION**

The polyamines putrescine, spermidine, and spermine are low molecular weight polycations that are critical for proliferation and differentiation of all living cells (14–16). Considerable evidence indicates that the activation of polyamine biosynthesis plays an important role in transformation induced by chemical carcinogens (17), viruses (18, 19), and oncogenes (20, 21). Induction of ODC overexpression using a transfection approach has been shown to induce transformation of fibroblasts and epithelial cells, particularly in the presence of mutated \( \text{ras} \) (22–24). The potential role of polyamines in breast cancer development is suggested by the presence of higher levels of these compounds and their biosynthetic enzymes in breast cancer specimens than in the surrounding normal tissue (25, 26).

Data from our and other laboratories have indicated that polyamines affect several steps in the hormonal control of breast cancer cell proliferation such as the binding of the ER to its responsive elements in DNA (27), the synthesis of \( \text{E}_2 \)-regulated cell cycle-specific genes (28), as well as the synthesis and action of hormonally regulated growth factors (29–31). Polyamines have also been shown to play an important role in the growth of hormone-independent tumors (1). Based on these observations, we hypothesized that the activation of polyamine biosynthesis may lead to the progression of breast cancer to a more aggressive phenotype. We have indeed observed that the induction of ODC overexpression in MCF-7 human breast cancer cells in culture results in a significant reduction in estrogen sensitivity (5). In addition, the induction of SAMDC overexpression in the same system led to increased clonogenicity in soft agar, indicative of a more aggressive biological behavior (32). Finally, using an \textit{in vivo} model of mammary tumor progression, we observed that elevated ODC activity was associated with the acquisition of a hormone-independent less-differentiated breast cancer phenotype (6).
However, despite this abundant experimental evidence, little information is currently available on the importance of polyamines in clinical breast cancer. Glikman et al. (33) observed that high ODC activity was associated with high cellularity, low histological differentiation, and high nuclear aplasia, thus suggesting but not proving the potential prognostic significance of tumor ODC levels. Kingsnorth et al. (34) showed that increased intracellular polyamine levels were positively correlated with factors known to have an adverse effect on survival such as high histological grade and ER-negative status. Furthermore, these authors reported that tumors which recurred within 2 years of mastectomy had significantly higher levels of spermidine and spermine than those that did not. However, a formal survival analysis that takes into account the contribution of other prognostic variables was not performed.

Our study demonstrates for the first time the prognostic significance of elevated ODC activity in clinical breast cancer. Despite the limited number of specimens analyzed, high ODC activity in primary tumors significantly predicted shorter disease-free and overall survival. Remarkably, with regard to the latter, ODC level was the most significant predictor among the variables tested, coming ahead of even the number of positive nodes. It should be noted that the statistical significance of the prognostic influence of ODC on survival was increased when breast cancer-specific mortality was included in the analysis, as opposed to death from any cause. This finding reinforces the specificity of the association between tumor ODC activity and risk of death from breast cancer. ER status was not found to be a significant factor in our analysis, possibly as a result of the limited sample size. This finding again emphasizes the important prognostic role of ODC, which seems to be distinctly superior to ER, a reasonably well-established prognostic factor in breast cancer (35, 36). Incidentally, ODC activity did not correlate with ER status, a finding also reported by Glikman et al. (33). Because enhanced polyamine biosynthesis is frequently associated with increased proliferation (15), it is possible that high ODC activity may reflect a large percentage of cells in S phase or the growth fraction of the tumor. Unfortunately, cell kinetic parameters to allow this analysis are not available in our specimens.

An interesting observation of our study is the preservation of the prognostic effect of ODC on survival after recurrence is included in the model, although this effect was of borderline statistical significance. In this analysis, the number of positive nodes lost its predictive value. This finding indicates that node positivity is primarily a predictor of earlier recurrence, which in turn is associated with shorter survival. In contrast, high ODC activity, in addition to predicting relapse, may identify a subgroup of breast cancers with particularly aggressive disease, leading to earlier demise after recurrence has occurred. This conclusion is reinforced by our analysis of survival from recurrence to death in the group of patients who relapsed and died of breast cancer. ODC activity was found to have a negative

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Evaluation of prognostic factors according to multivariate analysis</th>
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<tbody>
<tr>
<td>Variable</td>
<td>Disease-free survival</td>
</tr>
<tr>
<td></td>
<td>(39 patients, 20 recurrences)</td>
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<tr>
<td>Age</td>
<td>Risk ratio</td>
</tr>
<tr>
<td>ER</td>
<td>1.00</td>
</tr>
<tr>
<td>Number of positive nodes</td>
<td>1.14</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>1.20</td>
</tr>
<tr>
<td>log (ODC)</td>
<td>3.27</td>
</tr>
<tr>
<td>log (SSAT)</td>
<td>7.53</td>
</tr>
<tr>
<td>log (SAMDC)</td>
<td>0.59</td>
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<th>Table 4</th>
<th>Multivariate analysis of prognostic factors including recurrence as a time-dependent covariate</th>
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<tbody>
<tr>
<td>Variable</td>
<td>Overall survival</td>
</tr>
<tr>
<td>Recurrence</td>
<td>Risk ratio</td>
</tr>
<tr>
<td>Age</td>
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</tr>
<tr>
<td>ER</td>
<td>0.70</td>
</tr>
<tr>
<td>Number of positive nodes</td>
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<tr>
<td>Tumor size (cm)</td>
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</tr>
<tr>
<td>log (ODC)</td>
<td>0.98</td>
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<tr>
<td>log (SSAT)</td>
<td>2.01</td>
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<tr>
<td>log (SAMDC)</td>
<td>0.74</td>
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Fig. 3 Influence of a 10-fold difference in ODC activity (—, ODC = 10 pmol/mg; - - - - , ODC = 100 pmol/mg) on survival in our patient population after adjustments were introduced for ER and lymph nodal status.
influence on this end point, although the effect was again of only borderline significance. Analysis of a larger sample size is required to clarify this issue. It is important to recognize that due to the amount of tissue needed to perform all the enzyme measurements (≥1 g), only large primary tumors were included in our study (mean diameter, 4.52 cm ± 2.81 SD). Therefore, it remains to be established whether our findings also apply to smaller tumors, which are more frequently encountered nowadays as a result of increased public awareness of breast cancer and screening.

The prognostic value, if any, of SSAT expression is uncertain. Although SSAT level was predictive of shorter disease-free survival in the multivariate analysis, expression of this enzyme did not predict overall survival, a more important end point. SAMDC expression was not found to have any prognostic value in our study. Although considerably less information is available on the role of these two enzymes in tumor biology, they have both been the focus of active recent investigation testing their role as potential targets for chemotherapeutic intervention (39–41). Of interest, the expression of SAMDC and SSAT was also not found to identify tumors with aggressive behavior in our studies using an experimental model of breast cancer progression (6).

In summary, our data indicate that the level of ODC expression in primary breast cancer is a major prognostic factor with regard to disease-free survival and overall survival. These findings emphasize the likely biological importance of polyamines in clinical breast cancer and identify the polyamine metabolic pathway as a potential therapeutic target.

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