Methyl-p-hydroxyphenyllactate-esterase Activity in Breast Cancer: A Potentially New Prognostic Factor in Short-Term Follow-up

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ABSTRACT

We assayed methyl-p-hydroxyphenyllactate esterase (MeHPLAase) activity in 48 cases of primary breast cancer. MeHPLAase activity did not show significant correlation with estrogen receptor and progesterone receptor levels. No significant relationship was found between enzymatic activity and tumor diameter, lymph node status, mitotic activity, degree of nuclear differentiation, and proportion of the S-phase fraction. During the follow-up period (median, 18.8 months; range, 6—69 months), recurrences were observed in 18 of 48 (37%) cases. The Weibull survival regression model using the enzymatic activity as a continuous covariate showed that levels of enzymatic activity were directly associated with the risk of recurrence (P = 0.02). Assuming the mean value of enzymatic activity as the cutoff value, we found a statistically significant relationship between high MeHPLAase activity and shorter recurrence-free survival. On multivariate analysis, MeHPLAase activity proved to be an independent factor for predicting a short period of recurrence-free survival.

INTRODUCTION

A second binding site for estradiol, termed type II EBS (1), has been described in many normal and malignant tissues (2-6). This site has greater capacity and lower affinity for estradiol than classical ERs and binds bioflavonoid compounds such as quercetin, which has been demonstrated to inhibit cancer cell growth in many experimental systems (4, 7–11). Recently, it has been shown that the function of type II sites was to bind an endogenous ligand, probably derived from cell metabolism (12, 13), that was identified as MeHPLA (14–16). This endogenous ligand has a high binding affinity for type II sites (Kd = 4–5 nm). Although the precise mechanism involved in MeHPLA regulation of cell growth remains to be resolved, occupancy of nuclear type II sites by MeHPLA and related bioflavonoids inhibits estrogen stimulation of rat uterine growth and mammary tumor cell proliferation in vitro and in vivo (7, 16–18). MeHPLA hydrolysis driven by MeHPLAase gives rise to free HPLA, which has a much lower binding affinity for type II sites and is inactive in cell growth inhibition as compared with MeHPLA (7, 17). Therefore, changes in the MeHPLAase activity and consequently in HPLA levels may be involved in cell growth regulation in normal and neoplastic tissues.

It has been observed that human mammary cancers contain type II EBS (2), which can mediate the growth-inhibitory action of both endogenous ligand (MeHPLA) and chemically related bioflavonoids (10, 17). Furthermore, in rat uterus and mouse mammary tumors, the concentration of MeHPLA may be controlled by MeHPLAase (17, 19). In the rat uterus, this enzyme has been partially purified (>4300-fold; Ref. 20), and its activity has been shown to be under estrogen regulation (19, 20). Rat, mouse, and human mammary tumors are deficient in MeHPLA as compared to their normal counterparts (14, 15, 17, 19). One explanation for the observed deficiency of endogenous ligand in such tumors could be a high activity of MeHPLAase. Considering that human breast cancers contain both type II sites and MeHPLA, we decided to verify if the levels of MeHPLAase activity might play a role in the prognostic characterization of breast cancer patients.

PATIENTS AND METHODS

Patients. Forty-eight consecutive cases of infiltrating breast cancer were entered in this study. No selection criteria were used other than excluding in situ tumors and cases showing distant metastases at the moment of surgery. In particular, 17 patients (35%) were premenopausal, and 31 (65%) were postmenopausal (mean ± SD, 55.2 ± 14.3 years; median, 53.5 years; range, 28–83 years). Cases were classified following the pTNM classification. The mean tumoral diameter was 2.2 ± 1.2 cm (n = 48; median, 2.0 cm; range, 0.7–7.1 cm), as assessed by measuring the lesion with a brass caliper having an accuracy of one-tenth of a millimeter. Thus, cases comprised the T1–T3 groups. Forty-two percent of patients were node negative (n = 20), and 58% were node positive (n = 28). All patients were treated with locoregional radiotherapy after surgery (quadrantectomy for T1, mastectomy for T2–T3, both in association with axillary lymph node resection) by standard protocol. Histologically, all cases were invasive cancers, mainly ductal carcinomas. Outcome of the disease was available for each patient. The observation period ranged between 6 and 69 months (mean ± SD, 19.1 ± 9.2 months; median, 18.5 months). During follow-up, 18 of 48 (37%) patients had recurrence of disease (recurrence on scar, recurrence in residual glandular tissue, and distant metastasis), whereas 30 patients showed no relapse of disease (63%). Among the patients who relapsed, only two died of their disease. The length of time (months) between the primary treatment date and the last observation, with or without relapse of disease, was defined as RFS, and the occurrence of an event (relapse or no relapse) was registered. To screen for recurrence, a fixed follow-up schedule was used for all patients. The mean length of RFS was 17.3 ± 7.1 months (median, 16.5 months; range, 6–47 months). Because of the relatively short period of observation in the present study, we considered only RFS relative to the enzymatic activity.

Histological Characterization. For the histological classification of the tumors, basically, the recommendations of WHO were followed (21). Thirty-seven cases were ductal invasive carcinomas not otherwise specified (77%), and 11 cases were lobular invasive carcinomas (23%). In the latter group classical form, variants and pleomorphic type are included (22, 23).

ng. Representative well-fixed tumoral tissue specimens were used for histological examination. Five-μm sections stained with H&E were observed using a microscope for evaluation of the ng (24). Nuclear pleomorphism and nuclear features were estimated, and cases were scored ng1 (well-differentiated nuclei) to ng3 (poorly differentiated nuclei). Cases with a good or intermediate degree of differentiation (ng1 or ng2) were grouped together and compared with cases with poorly differentiated nuclei (ng3; Ref. 25).

Mitotic Activity. The mitotic activity (MAI) estimated in a defined area of histological section was considered. MAI was assessed as reported previously in the literature (26). A Laborlux S microscope (Leitz, Wetzlar, Germany) equipped with a ×400 objective and with Periplan ×10 GF eyepieces (field diameter, 0.45 mm; field of view index, 18) was used. One ×400 field is...
defined as a high-power field. The MAI is the number of mitoses counted in 10 high-power fields (1.59 mm² of histological section). We observed a wide variation in the number of observed mitoses (range, 1–23; mean ± SD, 5.2 ± 5.6).

ERs and PRs. ERs and PRs were assessed as reported previously (27) using the dextran-coated charcoal method, as recommended by the Italian Committee for Standardization of Tissue Hormonal Receptor Assays (28). Cases were scored receptor positive if amounts of ER or PR equal to or greater than 10 femtomol/mg cytosolic protein were found.

Flow Cytometry. Nuclear DNA content (DNA index) and evaluation of the tumoral proliferating fraction (SPF) were assessed on freshly obtained or frozen tumoral specimens in the same experimental session of ER and PR assays, using a RACOM System A flow cytometer (Ref. 29; Ylem, Avezzano, Italy). Tissues were minced in nuclear isolation medium-4’,6-diamidino-2-phenylindole (Ylem), a solution able to isolate and stain the nuclei in a single step. Data derived from the cytometric analysis were elaborated using MODFIT software (Verity Software House, Topsham, ME). In the present work, only the SPF was considered (mean ± SD, 7.6 ± 4.4; range, 0–17%). After separate analysis, the cutoff for SPF was set at 7% to define two groups with low or high SPF (≤7 or >7, respectively). A different cutoff was not adopted for diploid or aneuploid cases (30), because the group of patients studied was composed of both node-negative and node-positive cases, and the number of cases available was limited (12 cases were diploid, and 36 were aneuploid).

MeHPLAase assay. MeHPLA tritiated to the specific activity of 35.2 Ci/mmol was purchased from Amersham (Aylesbury, United Kingdom). HPLA and steroid hormones were purchased from Sigma (St. Louis, MO), and quercetin (3,3’,4’,5,7-pentahydroxyflavone) was obtained from Aldrich (Steinheim, Germany). Enzymatic activity was measured according to the method described by Markaverich et al. (19). Cytosol fractions were prepared by ultracentrifuging freshly obtained tumor fragments as described previously (31). Cytosol aliquots (0.5 ml at a protein concentration of 1 mg/ml) were incubated with [3H]MeHPLA (10 pmol/ml) for 8 min at 37°C at pH 7.4. Preliminary experiments revealed that MeHPLAase activity was linear in the time range between 2 and 10 min and in a range of protein concentrations between 0.5 and 4 mg/ml. Competitors were used in DMSO, because [3H]MeHPLA hydrolysis was markedly inhibited by ethanol. After incubation, the reaction mixture (pH = 7.4) was first extracted three times with 3 volumes of ethyl acetate to obtain fractions containing [3H]MeHPLA and then acidified (pH 1.0) with HCl and extracted three times with 3 volumes of ethyl acetate to obtain [3H]HPLA. In experiments aimed at confirming the hydrolysis of MeHPLA from neutral and from acidified fractions. Results were expressed as pico moles of [3H]MeHPLA hydrolyzed per milligram of cytosolic protein per minute (pmol/mg p/min). The characteristics of MeHPLAase activity in breast tumors were similar to those observed previously in rat uterus (19) and human ovarian tumors (31) in that: (a) it is very sensitive to ethanol; (b) it is almost totally destroyed by heating; and (c) it is inhibited by quercetin (data not shown). For prognostic evaluation, a cutoff of 1.07 corresponding to the mean value of MeHPLAase activity distribution was chosen to distinguish patients with low (≤ 1) from those with high (>1) esterase activity.

Statistical Analysis. Correlations between ER, PR, and MeHPLAase activity were assessed by the Spearman rank-correlation test. The Mann-Whitney nonparametric test was used to analyze the distribution of MeHPLAase activity according to various clinicopathological parameters. The fully parametric survival regression approach was used, using MeHPLAase activity as a continuous covariate. Specifically, the RFS times of the patients were empirically verified by hazards plot to follow a Weibull distribution (32). The method of maximum likelihood was then used to estimate the parameters in the Weibull survival regression model. All medians and life tables were computed using the product-limit estimate by Kaplan and Meier (33), and the curves were examined by means of the log-rank test (34). Univariate and multivariate analyses were performed using the Cox proportional hazards model (35).

RESULTS

The distribution of MeHPLAase activity in 48 cases of breast cancer seemed to be skewed toward the lower values (mean ± SD, 1.071 ± 0.88; median, 0.83; range, 0.21–5.19). Thirty cases were ER positive (62%) and 18 cases were ER negative (38%), whereas 33 cases were PR positive (69%) and 15 cases were PR negative (31%). ER and PR status (≥10 or <10 femtomol/mg cytosolic protein), as analyzed by the product-limit estimate according to Kaplan and Meier, did not prove to be significantly predictive of recurrence in this patient series. MeHPLAase activity did not show any correlation with ER and PR, and it did not show any significant difference between tumors of pre- and postmenopausal patients (data not shown).

Table 1 shows the distribution of MeHPLAase activity according to the clinicopathological characteristics of primary breast cancers. No significant relationship was observed between MeHPLAase activity and MAI, lymph node status, tumor diameter, ng, and SPF.

The Weibull survival regression model using the enzymatic activity as a continuous covariate showed that the levels of enzymatic activity were directly associated with the risk of recurrence (χ² = 5.18; P = 0.02). Fig. 1 shows the plot of estimate of RFS as a function of the levels of MeHPLAase activity. At the 2-year follow-up, the
Table 2 Univariate and multivariate analysis of prognostic variables for RFS in 48 primary breast cancer patients

<table>
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<th>Covariate</th>
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<th>Multivariate</th>
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<td></td>
<td>RR1a</td>
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<tr>
<td>MeHPLAase activity status</td>
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<tr>
<td>High</td>
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<tr>
<td>&gt;2 cm</td>
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<td>2.05–27.36</td>
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<tr>
<td>Lymph node status</td>
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</tr>
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<td>0.59–4.08</td>
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a Unadjusted relative risk.
b Relative risk taking into account all the variables in the table.
c As assessed by flow cytometry on fresh tissue.

DISCUSSION

This report describes for the first time the presence and characteristics of MeHPLAase activity in primary breast cancer. The characteristics of MeHPLAase were similar to those described in ovarian cancer (31) relative to the high sensitivity of enzymatic activity to ethanol (and heating) and the susceptibility to quercetin inhibition. The enzymatic activity was higher in breast cancers (median, 0.83; range, 0.21–5.19) than in ovarian cancers (median, 0.062; range, 0–0.429; Ref. 31). Moreover, in contrast to what was observed in ovarian cancers, in breast tumors, MeHPLAase activity did not correlate with either ER or PR level. High levels of MeHPLAase activity (>1 pmol/mg p/mn) were associated in breast cancers with a more aggressive behavior in terms of RFS. These findings are the opposite of those observed in ovarian cancers (31). Taken together, these observations suggest that MeHPLAase activity can be regulated in a tissue-specific manner.

Because MeHPLAase metabolizes the endogenous type II EBS ligand displaying cell growth-inhibitory activity, it was consistent with this model of cell growth regulation that high enzymatic activity in breast cancers was associated with a more aggressive tumor behavior.

In normal rat uterus stimulated to growth by estrogen, MeHPLAase activity is up-regulated (19, 20). However, we failed to observe any significant difference in MeHPLAase activity between ER-positive and ER-negative cases. This probably reflects a situation of loss of normal regulatory mechanisms in tumor cells. This possibility is further supported by the lack of correlation between enzymatic activity and MAI or SPF (Table 1).

Despite radical surgery, many breast cancer patients are at risk of disease progression. Chemotherapy may significantly improve the prognosis in operated high-risk patients. Lymph node status is traditionally used as a criterion to select high-risk (i.e., lymph node-

activity compared to 38% (95% CI; 10–65%) for patients with endotumoral high (>1 pmol/mg p/min) enzymatic activity ($P = 0.02$).

In Table 2, univariate and multivariate analyses of prognostic variables for RFS are shown. Cases with high MeHPLAase activity, tumor diameter greater than 2 cm, and high MAI showed a significantly increased risk of recurrence. In the multivariate analysis, MeHPLAase activity retained an independent negative prognostic significance.
positive) patients, but evidences are reported in the literature that ACT can be beneficial in certain patients with breast cancer without axillary lymph node involvement (36). Clinicians often face the dilemma of whether to treat or not treat patients with tumors up or around 2 cm in diameter, especially those without axillary lymph node involvement (36), with ACT. High MeHPLAase activity resulted as a good marker in the nucleus of normal and malignant human tissue: characteristics of the multiple nuclear binding sites. Cancer Res., 42: 4449–4454, 1982.


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