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

Arnaldo Carbone,1 Fabio Giosuè Serra, Gabriella Ferrandina, Giovanni Scambia, Daniela Terrible, Rocco Bellantone, Mauro Piantelli, and Franco Oreste Ranelletti

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 EBS3 (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 recorded. 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...
Chromatograms were developed in hexane:ethyl acetate (1:1, vlv), and plates
Ci/mmol was purchased from Amersham (Aylesbury, United Kingdom).

obtain [3H]HPLA. In experiments aimed at confirming the hydrolysis of
of ethyl acetate to obtain fractions containing [3H]MeHPLA and then acidified
and 0.01, respectively. The same Rf values were obtained with unlabeled
gave a single peak of radioactivity with relative front of migration (Rf) of 0.41

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 RATCOM 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/mm mol 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 concentra-
tions 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 to HPLA, the ethyl acetate extracts and MeHPLA and HPLA standards
were dried under N2, resuspended in 50 μl of ethyl acetate, and
analyzed by TLC on activated silica gel plates (Merck, Darmstadt, Germany).
Chromatograms were developed in hexane:ethyl acetate (1:1, vlv), and plates
were scanned for radioactivity with a Bioscan System 200 Imaging Scanner
(Packard). The ethyl acetate extracts from neutral and from acidified samples
served a single peak of radioactivity with relative front of migration (Rf) of 0.41
and 0.01, respectively. The same Rf values were obtained with unlabeled
MeHPLA and with HPLA standards. After it had been demonstrated that
[3H]HPLA was the only metabolite of [3H]MeHPLA hydrolysis, the assay was
incubated on the basis of selective ethyl acetate extraction of [3H]MeHPLA
from neutral fractions and of [3H]HPLA from acidified fractions. Results were
expressed as picomoles of [3H]MeHPLA hydrolyzed per milligram of cyto-
solic 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 ac-
tivity 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 con-
tinuous 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 exam-
ined 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>
<thead>
<tr>
<th>Parameter</th>
<th>N⁵</th>
<th>Mean</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>48</td>
<td>1.071</td>
<td>0.830</td>
<td>0.210–5.190</td>
</tr>
<tr>
<td>Tumor diameter</td>
<td>27</td>
<td>1.210</td>
<td>0.810</td>
<td>0.300–5.190</td>
</tr>
<tr>
<td>≤2 cm</td>
<td>21</td>
<td>0.882</td>
<td>0.890</td>
<td>0.210–1.850</td>
</tr>
<tr>
<td>&gt;2 cm</td>
<td>21</td>
<td>1.163</td>
<td>0.810</td>
<td>0.210–5.190</td>
</tr>
<tr>
<td>Lymph node status</td>
<td>20</td>
<td>0.966</td>
<td>0.830</td>
<td>0.230–4.050</td>
</tr>
<tr>
<td>Negative</td>
<td>28</td>
<td>1.069</td>
<td>0.780</td>
<td>0.230–5.190</td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>1.081</td>
<td>1.130</td>
<td>0.210–1.640</td>
</tr>
<tr>
<td>NG</td>
<td>32</td>
<td>1.143</td>
<td>0.810</td>
<td>0.210–5.190</td>
</tr>
<tr>
<td>1 + 2</td>
<td>16</td>
<td>0.929</td>
<td>0.890</td>
<td>0.300–1.770</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>1.320</td>
<td>1.120</td>
<td>0.300–5.190</td>
</tr>
<tr>
<td>SPF⁶</td>
<td>29</td>
<td>0.976</td>
<td>0.760</td>
<td>0.230–4.050</td>
</tr>
</tbody>
</table>

⁵ Number of observations.
⁶ Assessed by flow cytometry on fresh tissue.

5407
Table 2 Univariate and multivariate analysis of prognostic variables for RFS in 48 primary breast cancer patients

<table>
<thead>
<tr>
<th>Covariate</th>
<th>RR1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>95% CI</th>
<th>χ²</th>
<th>P</th>
<th>RR2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>95% CI</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeHPLAase activity status Low</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>2.86</td>
<td>1.09-7.49</td>
<td>4.56</td>
<td>0.033</td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor diameter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2 cm</td>
<td>2.86</td>
<td>1.09-7.49</td>
<td>4.56</td>
<td>0.033</td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2 cm</td>
<td>7.49</td>
<td>2.05-27.36</td>
<td>9.298</td>
<td>0.0023</td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1.10</td>
<td>0.38-3.20</td>
<td>0.030</td>
<td>0.863</td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAI &lt;10</td>
<td>1.10</td>
<td>0.38-3.20</td>
<td>0.030</td>
<td>0.863</td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥10</td>
<td>3.80</td>
<td>1.41-10.23</td>
<td>6.979</td>
<td>0.0082</td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ng 1</td>
<td>1.10</td>
<td>0.38-3.20</td>
<td>0.030</td>
<td>0.863</td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPF&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (≤7)</td>
<td>1.55</td>
<td>0.59-4.08</td>
<td>0.784</td>
<td>0.376</td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High (&gt;7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.09-180.6</td>
<td>1.09-180.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR1&lt;sup&gt;a&lt;/sup&gt; = Unadjusted relative risk.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR2&lt;sup&gt;b&lt;/sup&gt; = Relative risk taking into account all the variables in the table.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPF = As assessed by flow cytometry on fresh tissue.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


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

Arnaldo Carbone, Fabio Giosuè Serra, Gabriella Ferrandina, et al.


Updated version  Access the most recent version of this article at: [http://cancerres.aacrjournals.org/content/57/23/5406](http://cancerres.aacrjournals.org/content/57/23/5406)

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.