ESTROGEN RECEPTOR α VARIANT PROTEIN LACKING THE HORMONE-BINDING PERSISTS IN HUMAN BREAST CANCER

Masao Hori, Jiro Shimazaki, Satoshi Inagawa, Masayuki Itabashi

Department of Pathology
Ibaraki Prefectural Central Hospital and Cancer Center
Ibaraki, Japan
(Received on Sept. 27, 1999, accepted on Nov. 12, 1999)

Key Words: Breast cancer, estrogen receptor α, variant protein.
Subjects: Patients.

Abstract

Estrogen receptor alpha (ERα) variant proteins lacking the hormone-binding domains translated from their splice variant mRNAs were examined by Western blot analysis of 80 cases of human breast cancer. Wild ERα protein (ERwtP) was expressed in 68 cases (85.0%), and of these cases, 40 (58.8%) co-expressed ERα variant protein corresponding in size to 41kDa (ERd5P). There was a significant correlation between co-expression of ERd5P and the splice variant mRNA lacking exon 5 (ERd5M) examined by reverse transcription-polymerase chain reaction. No protein bands for ERα variant corresponding in size to 52kDa (ERd7P) were observed in spite of the splice variant mRNA lacking exon 7 (ERd7M) being co-expressed with wild-type mRNA. Our results suggest that ERd5P persists and is stable in a large proportion of samples of human breast cancer tissue, whereas ERd7P is not. The functional difference between ERwtP and ERd5P has yet to be established, and its discovery may demonstrate the importance of ERd5P for anti-estrogen resistance in human breast cancer.

Introduction

It is well known that estrogen and its receptor are significantly related to the development and growth of human breast cancer; advanced studies about the receptor have been made possible since the success of cDNA cloning of the estrogen receptor α (ERα) (Green, Walter, et al., 1986). However, the problem of anti-estrogen resistance in human breast cancer remains to be established. In order to clarify the mechanisms of this resistance, studies of the structural and functional abnormalities of ERα itself are important, although the examination of several factors associated with ER, such as co-
factors, are also required. Several ERα variant mRNAs such as ERd5M and ERd7M are present in various cell lines of human cancer and are considered to be one reason for the deterrent to the normal function of ERα (Castles, Klotz, et al., 1995; Fuqua, 1994). Although we have already demonstrated that the expression of ERα splice variant mRNAs is not uncommon in human breast disease (Hori, Katano, et al., 1998), it is not clear whether the corresponding variant proteins persist and are stable. In the present study, we examined the distribution of variant ERα protein translated from splice variant mRNA in human breast cancer tissue. Our current investigation is directed at identifying ERd5P as well as ERd5M.

Materials and Methods

Specimens

Frozen tissues of invasive ductal carcinoma were obtained from 80 female patients, and the MCF-7 cell line (Dainippon Pharmaceutical Co, Ltd, Osaka, Japan) was used as a positive control for wild ERα.

Western blot analysis

Cell lysis and Western blot analysis of each sample and cell line was performed as described previously (Sambrook, Fritsch, 1989). The protein lyzate was electrophoresed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitro-cellulose membranes. After blocking with Tris-buffered saline, the filters were probed using antibody recognizing the amino terminal of ERα (ER-1D5, M7047; DAKO Japan, Kyoto, Japan). The specific complex was detected by the enhanced chemiluminescence Western blot protocol using an ECL detection kit (RPN2209; Amersham, Aylesbury, UK).

Enzyme immunoassay (EIA) procedure

Using powdered and homogenized samples of ductal carcinoma tissue, ERα protein was measured using a commercial EIA kit (Abbott Diagnostic Laboratories, North Chicago, IL, USA), as described elsewhere (Saccani-Jotti, Johnston, Salter et al., 1994). Values of over 14.0 fmol/mg protein were considered to be positive.

Reverse transcription polymerase chain reaction (RT-PCR) procedure

A part of each sample was homogenized and the total RNA was isolated using
total RNA isolation reagent (Trizol; Life Technologies, MD, USA) according to the manufacturer's instructions. RT and PCR parameters and the primer sets used to detect exon 5- and 7 deleted ERα mRNAs have been described previously (Hori, Katano, et al., 1998). The internal control mRNA of each specimen was demonstrated by carrying out the above procedure using a primer pair for β-actin and a commercial kit (Human β-actin Control Panel; Clontech, Palo Alto, CA, USA). The amplified products were electrophoresed using 2.5% (w/v) agarose gels, and stained with ethidium bromide.

Statistical analysis

Any correlation was evaluated by the Fisher's exact test, and those that reached the p <0.05 level were considered significant.

Results

Expression of wild and variant ERα protein

Western blot analysis showed that of the 80 tissue samples, wild ERα protein (ERwtP) corresponding in size to 67kDa was expressed in 68 cases (85.0%) and in the MCF-7 cell line. 40 of these 68 cases (58.8%) and MCF-7 co-expressed ERα variant protein corresponding in size to 41kDa (ERd5P). None of the cases expressed ERd5P alone (Figure 1). No 52kDa protein bands for ERα variant protein (ERd7P) were observed.

Correlation between ERα value and the co-expression of ERd5P

The ERα values (fmol/mg protein) of the 80 tumor samples measured by EIA ranged from 2.0 to 741.0, and 49 of the cases (61.3%) were judged positive. There was no significant correlation between ERα positivity and the expression of ERwtP detected by Western blotting, and 23 out of the 31 cases that were judged negative for ERα expressed ERwtP (Table I). In the 68 cases that expressed ERwtP, there was a significant difference in ERα positivity between the group that exhibited ERd5P co-expression and that which expressed ERwtP alone (Table II).

Correlation between co-expression of ERα splice variant mRNA and ERd5P

All of the 80 samples and the MCF-7 cell line exhibited 479-base-pair (bp) signals that were detectable with primers specific for β-actin. Using the exon 5 and exon 7 primer sets for ERα cDNA, PCR products were detected in 71 and 54 of the samples,
Figure 1.: The expression of estrogen receptor α (ERα) proteins in human tissue samples, as demonstrated by Western blot analysis. ERα variant proteins corresponding in size to approximately 41 kDa (ERd5P) were co-expressed with wild proteins corresponding in size to approximately 67 kDa (lanes 1, 2). No 52 kDa protein bands for ERα variant protein (ERd7P) were observed. There was no band corresponding in size to ERd5P in case of breast cancer that expressed ERwt5M alone by RT-PCR (lane 3).

Figure 2.: The expression of ERα mRNAs in human tissue samples, as demonstrated by the reverse transcription-polymerase chain reaction method. ERd7M corresponding in size to 484 base pairs (bp) was co-expressed with ERwt7M corresponding in size to 668 bp (lanes 1, 2, 4, 6).
### TABLE I
Correlation between the expression of ERwtP and ER-EIA result

<table>
<thead>
<tr>
<th></th>
<th>ERwtP expression</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>ER-EIA positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(≥14.0 fmol/mgP)</td>
<td>42.00</td>
<td>7.00</td>
</tr>
<tr>
<td>ER-EIA negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&lt;14.0 fmol/mgP)</td>
<td>26.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Total</td>
<td>68.00</td>
<td>12.00</td>
</tr>
</tbody>
</table>

### TABLE II
Correlation between the co-expression of ERd5P and ER-EIA result (p<0.001)

<table>
<thead>
<tr>
<th></th>
<th>ERwtP expression</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>ER-EIA positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(≥14.0 fmol/mgP)</td>
<td>37.00</td>
<td>8.00</td>
</tr>
<tr>
<td>ER-EIA negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&lt;14.0 fmol/mgP)</td>
<td>3.00</td>
<td>20.00</td>
</tr>
<tr>
<td>Total</td>
<td>40.00</td>
<td>28.00</td>
</tr>
</tbody>
</table>

### TABLE III
Correlation between the co-expression of ERd5P and Erd5M (p<0.001)

<table>
<thead>
<tr>
<th></th>
<th>ERwtP expression</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>Erd5M co-expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>39.00</td>
<td>9.00</td>
</tr>
<tr>
<td>Negative</td>
<td>1.00</td>
<td>19.00</td>
</tr>
<tr>
<td>Total</td>
<td>40.00</td>
<td>28.00</td>
</tr>
</tbody>
</table>
respectively. All of the bands that corresponded in size with splice variants (ERd5M, 344 bp; ERd7M, 484 bp) were observed with bands that corresponded to the wild mRNA (ERwt5M, 483 bp; ERwt7M, 668 bp), and none of the cases exhibited isolated splice variants (Figure 2). ERd5M and ERd7M co-expression was detected in 48 of 71 cases (67.6%) and 40 of 54 cases (74.1%), respectively. MCF-7 cells co-expressed either ERd5M or ERd7M. In the 68 cases that expressed ERwtP, there was a significant correlation between co-expression of ERd5M and ERd5P (Table III).

Discussion

The human ERα gene is located on the long arm of chromosome 6 and has 8 exons (Iwase and Kobayashi 1997). The corresponding protein has six conserved domains. Several ERα variant mRNAs such as ERd5M and ERd7M are present in various cell lines of human cancer and are considered to be one reason for the deterrent to the normal function of ERα (Castles, Klotz, et al., 1995; Fuqua, 1994). Although we have already demonstrated that the expression of ERα splice variant mRNAs is not uncommon in human breast disease (Hori, Katano et al., 1998), it is unclear whether the corresponding variant proteins persist and are stable. Madsen, Reiter et al. (1997) attempted to detect the variant proteins using the MCF-7 cell line and several anti-estrogen resistant cell lines. Since the expression of ERd5P and ERd7P was not detected in any of these cell lines although ERd5M and ERd7M were detected, Madsen, Reiter et al. (1997) suggested that variant ERα protein would be less stable and would disappear after translation from its mRNA. However, that variant proteins persist in human breast cancer tissue was expected from our previous study using an immunohistochemical assay using two monoclonal antibodies, that recognize either the amino or carboxyl terminal (Hori, Iwasaki et al., 1999). In the present study, as ERd5P expression and the concordance with ERd5M expression was confirmed by Western blot and RT-PCR analysis, the existence of ERd5P translated from ERd5M in human breast cancer was demonstrated. Although we agreed with Madsen, Reiter et al. (1997) about the lack of ERd7P, a large number of all variant proteins that we found in the previous study involved ERd5P. Since MCF-7 cells also exhibited ERd5P and ERα-negative tissue samples (as determined by EIA) frequently showed signals when subjected to Western
blot analysis, there is likely to have been a difference in the sensitivity of detection between the protocols of Madsen, Reiter et al. (1997) and ourselves.

EIA is a common method for the measurement of ERα protein. However, since the EIA we used recognizes the amino terminal of the protein, there is a good possibility that we not only measure the wild, but also the variant proteins. The ERα-EIA-positivity rate was higher in the cases with co-expression of ERd5P than those with the expression of ERwtP alone, and this result suggests that the protein detected by our EIA contains a considerable amount of ERd5P. When measuring ERα levels using EIA, the domains that the antibody recognizes should always be considered.

The expression of splice variant mRNAs such as ERd5M and ERd7M is not specific to breast cancer tissue, and their expression is also detected in ERα-positive cancer cell lines (Hori, Katano et al., 1998). Gallacchi, Schoumacher et al. (1998) reported that the proportion of ERd5M in total ERα mRNA was higher in recurrent cancer than in primary breast cancer. Fujimoto, Ichigo et al. (1997) suggested that ERd5M abundance might be a useful indicator of metastatic potency in gynecological cancers. However, it is unclear whether these aberrant forms correlate directly with the anti-estrogen resistance of breast cancer. From the viewpoint of mRNA levels, our results did not support clearly the correlation between splice variants and anti-estrogen resistance (Hori, Katano et al., 1998). However, from the viewpoint of protein levels, the present results showed that ERd5P translated from ERd5M persisted in many cases, whereas ERd7P did not and later became less stable. Although we did not examine any other splice variant mRNAs and could not deny the presence of extremely low amounts of any other variant proteins, our results suggest that ERd5P is the main variant protein that persists in human breast cancer. Although several authors have pointed out the abnormal functions of splice variant mRNAs (Chaidarun and Alexander 1998; Rea and Parker 1996; Wang and Miksicek 1991), further study is required to pursue the functional difference between ERwtP and ERd5P. The results of such a study may demonstrate the importance of ERd5P for anti-estrogen resistance in human breast cancer.

Acknowledgment

This work was supported in part by a Grant-in-Aid for the encouragement of research from the Annals of Cancer Research and Therapy.
References


COPYRIGHT © 2000 BY
PJD PUBLICATIONS LIMITED, P.O. BOX 966, WESTBURY, NY 11590

96