Association of ARNT Splice Variants with Estrogen Receptor-negative Breast Cancer, Poor Induction of Vascular Endothelial Growth Factor under Hypoxia, and Poor Prognosis

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ABSTRACT

The aryl hydrocarbon receptor nuclear translocator (ARNT) is a basic helix-loop-helix transcription factor that forms heterodimers with the aryl hydrocarbon receptor (AhR) or hypoxia inducible factor-1α to activate transcription via xenobiotic response element or hypoxia response element, respectively. Thus, it plays a major role in two key biochemical pathways involved in tumor growth. We previously showed that estrogen receptor (ER)-negative breast cancer cell lines expressed a splice variant of ARNT that was associated with Ah nonresponsiveness. We have now used a sensitive PCR method to analyze the expression of the variant in a series of 92 breast cancers to assess interactions with the ER and prognosis. The splice variant could be detected in all of the cases examined, with high ratios of variant:full-length ARNT (≥10) characterized in 10 cases. When the patient group was split into quartiles by increasing splice variant ratios, there was an inverse relationship of ER status to ARNT splice-variant ratios (P = 0.01, χ²). Univariate analysis showed that cases with high ARNT splice-variant ratios ≥10 had a worse relapse-free and overall survival (P ≥ 0.03; hazard ratio, 2.7; and P = 0.006; hazard ratio, 3.9, respectively). In multivariate analysis for relapse-free and overall survival, ARNT splice-variant ratio was the strongest independent factor and, although inversely related to ER, remained a separate risk factor. At least two potential mechanisms could explain this phenomenon: the loss of aryl hydrocarbon receptor-mediated antiestrogenic activity or the blockade of a proapoptotic pathway induced by hypoxia. Because several enzymes involved in drug resistance are induced through a xenobiotic response element, the tumors presenting high ARNT splice-variant ratios may be specifically targeted by drugs normally degraded or inactivated. This study shows the biological importance of ARNT splice variants in the behavior of human breast cancer and suggests that the breast cell lines in which the splice variant was discovered may be useful models for further investigation.

INTRODUCTION

Angiogenesis, induced by hypoxia and responsiveness to the tumor promoter dioxin, share biochemical pathways regulated by the ARNT protein. In the case of angiogenesis, the HIF-1α is stabilized in response to decreased oxygen availability and heterodimerises with ARNT/HIF-1β to regulate specific DNA sequences that contain hypoxia response elements (1, 2). The genes regulated by these elements include VEGF, a key angiogenic factor, and also glycolytic enzymes (3). The response to dioxin is mediated by its receptor, the AhR, which heterodimerises with ARNT/HIF-1β to regulate genes via the binding to xenobiotic response elements (4). ARNT/HIF-1β is involved in the induction of two distinct pathways, which could lead to a nuclear competition for HIF-1α recruitment in cells that express functional ARNT. Thus, changes in ARNT/HIF-1β expression or function may be relevant to two major areas of tumor biology.

Previous studies have reported in human breast cancer cell lines a direct correlation between ER expression and the AhR responsiveness (5–12). Recently, we showed that MDA-MB-231, Adriamycin-resistant MCF7, and MDA-MB-435 ER negative cell lines did not respond to dioxin and expressed predominantly a new splice variant of ARNT, which lacked the COOH-terminal transactivating domain and which acted in a dominant-negative fashion (13). This mechanism has been shown to be associated with the ER, because AhR responsiveness was restored in MDA-MB-231 cells by transient transfection of human ER (10, 14). Because a cell proliferation inhibi-

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3 The abbreviations used are: dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin; ARNT, Ahr nuclear translocator; AhR, aryl hydrocarbon receptor; HIF, hypoxia-inducible transcription factor; VEGF, vascular endothelial growth factor; ER, estrogen receptor; RT-PCR, reverse transcription-PCR; EGFR, epidermal growth factor receptor; RFS, relapse-free survival; OS, overall survival.
The hypothalamic-pituitary-adrenal axis regulates the production of ACTH, which stimulates the adrenal cortex to produce cortisol. This regulatory pathway has been described to involve AhR independently of dioxin, the ARNT splice variant could have a function in enhancing tumor progression (15).

However, because ARNT/HIF-1β is implicated in the activation of VEGF expression under low oxygen condition, the splice variant may be associated with poor induction of hypoxia-regulated genes. Therefore, two human breast cancer cell lines exhibiting the ARNT splice variant were compared with an ER-positive and an ER-negative cell line lacking the splice variant, to study their ability to induce VEGF mRNA under a low oxygen environment.

Whether ARNT splice variants exist in primary human breast cancer and the relationship to clinical behavior of tumors is unknown. Therefore, we have examined 92 primary breast cancers for the presence of ARNT splice variants and their correlation with ER expression, and prognosis.

MATERIALS AND METHODS

Cell Culture. The breast carcinoma cell lines T-47D, MDA-MB-231, MDA-MB-435, and MDA-MB-468 were obtained from the American Type Culture Collection (Rockville, MD) and serially passaged using 0.25% trypsin-0.02% EDTA. All of the cell lines were cultured in DMEM (Clare Hall Laboratories, Imperial Cancer Research Fund) supplemented with 10% FCS and 4 mM glutamine. For all of the experiments, equal densities as opposed to equal numbers of cells were chosen to compensate for size differences. Cells were plated onto 100-mm or 150-mm Petri dishes before experiments in such a way that they were subconfluent at the start of each experiment. Culture medium was replaced 16 h prior to exposure to experimental conditions. Cells were routinely cultured in 95% O₂ and 5% CO₂ at 37°C and were made hypoxic by placing them in a Heto-Holten Cell house 170 incubator (Heto-Holten) at 0.1% O₂, 2% CO₂, and balance nitrogen. Desferrioxamine (DFO) was added to the culture medium prior to incubation at a final concentration of 100 μM.

RNA Analysis. Total RNA was extracted using TRI Reagent (Sigma, Poole, United Kingdom). RNase protection assays were performed essentially as described previously (16, 17), with parallel hybridization using 10 μg of RNA for VEGF₁ ᵃ and 1 μg for U6 small nuclear RNA (used as an internal loading control). ³²P-labeled riboprobe were generated using SP6, T3, or T7 RNA polymerase. The templates used yielding protected fragments as follows: 517 bp for VEGF₁ ᵃ (X62568) and 106 bp for U6 (X01366). After resolution on 8% polyacrylamide gels, quantification was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and each signal was standardized to the internal control.

Using this probe and upper band for the 121 amino acid (aa) form of VEGF, we generated a lower band for the 165 and 189 aa forms. Each experiment was performed three times in duplicate.

Patient Characteristics. Patients were treated by simple mastectomy or lumpectomy and radiotherapy, with axillary node sampling. Patient follow-up was conducted every 3 months for the first 18 months, every 6 months for the next 18 months, and yearly thereafter. Patients with confirmed recurrent disease were treated by endocrine therapy for soft tissue or skeletal disease or by chemotherapy for visceral disease or failed endocrine therapy. Patients with isolated soft tissue relapse also received radiotherapy. All node-positive premenopausal patients and those positive postmenopausal patients with a tumor size >3 cm or with grade III and ER-negative tumors received six courses of i.v. chemotherapy (cyclophosphamide, methotrexate, and 5-flouracil). Tamoxifen was given to premenopausal patients with ER-positive tumors and to all postmenopausal women for 5 years.

RT-PCR Method for ARNT. RNA samples (300–1000 ng) from breast cancer patients were reverse transcribed at 42°C for 30 min in 20 μl of RT mixture that contained 1× PCR buffer, 2.5 mM MgCl₂, 1.0 mM dNTPs, 1 unit/μl RNase inhibitor, 2.5 units/μl reverse transcriptase, and 2.5 μM oligo d(T). After transcription, the reaction mixture was incubated at 99°C for 5 min to inactivate enzymatic activity. The transcribed cDNAs were amplified in 50 μl of PCR mixture that contained 1× PCR buffer, 2.0 mM MgCl₂, 0.2 mM dNTP, 2.5 units/50 μl Ampli Taq polymerase, and 10 μM both reverse primer and ³²P[ATP]-labeled forward primers. The PCR thermal profile used was: initial denaturation at 95°C for 2 min, 26 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1.5 min), followed by final extension at 72°C for 7 min. The Perkin-Elmer PCR reagents and machine were purchased from Roche Molecular Systems, Inc. (Branchburg, NJ). PCR products were separated on 5% polyacrylamide gel and exposed to phosphor-screen overnight. Storm 860 (Molecular Dynamics, Inc.) was used to quantify intensities of the bands. The normal PCR product of ARNT is ~2.4 kb, and the spliced variant was ~1.1 kb, as described previously (13). The primers used were: ARNT forward primer, 5’-ACT GCC AAC CCC GAA ATG AC-3’; and ARNT reverse primer, 5’-CTC CCC CAC CCC TTA GCC TC-3’.

EGFR and ER Assays. ER content was determined using an ELISA technique (Abbott Laboratories). Tumors were considered positive when cytosolic ER levels were greater than 10 fmol/mg cytosolic protein. Receptor values were monitored by participation in the EORTC quality control scheme. EGFR was measured using ligand binding of ¹²⁵I-labeled EGF to crude plasma membrane fractions (18). Receptor values greater than 20 fmol/mg membrane protein were taken as positive.

Statistical Analysis. Spearman rank correlation was used for continuous variables and χ² test for analysis of results split categorically. The survival analyses were done using Kaplan-Meier statistics and Cox multivariate analysis for assessment of the individual role of multiple factors.

RESULTS

ARNT Splice Variant and Hypoxic Induction of Human Breast Cancer Cell Lines. MDA-MB-231 and MDA-MB-435 ER-negative human breast cancer cell lines have been previously reported with the highest ratio of ARNT splice variant:full length (13). To assess the potential impact of the ARNT splice-variant expression pattern on VEGF mRNA induction under hypoxia, several human breast cancer cell lines were compared by RNase protection assay according to their ability to express the ER (Fig. 1). The level of VEGF mRNA under 0.1% O₂ was increased by 1.4- and 1.8-fold, respectively.
in MDA-MB-231 and MDA-MB-435 cell lines (ER negative), in contrast to T47D (ER positive) and MDA-MB-468 (ER negative), which were inducible by 13.8- and 3.5-fold, respectively, under the same conditions. The basal level of VEGF mRNA was highest in MDA-MB-231 cell line known to present a ras mutation, which can be involved in the regulation of VEGF expression (19, 20). This result showed that the two ER-negative cell lines exhibiting the ARNT splice variant had the higher basal level of VEGF mRNA expression and the lowest induction under hypoxic conditions. To evaluate whether such ARNT splice variants exist in primary tumors and whether there was any relationship to ER status or prognosis of the cancers, we studied a series of breast cancer patients.

**Clinical Study.** Ninety-two patients were studied for expression of ARNT splice variants compared with other pathological variables, including ER, EGFR size, grade, and node status. Clinical details are summarized in Table 1.

**Analysis of ARNT Splice-Variant Ratio.** RNA samples from these 92 cases were analyzed by RT-PCR, and transcripts corresponding to the wild-type and truncated ARNT were observed at 2.4 and 1.1 kb as illustrated in Fig. 2, showing results for 16 patients. Ratios for the two transcripts were determined by densitometry, and the results showed that the ratio varied from 0.1 to 32.3 (median, 2.3).

**Relationship of ARNT Splice Variants to ER and Other Variables.** ARNT splice variant:full length ratio was analyzed taking the cases with highest ratio >10, and also as a continuous variable and splitting by the median. In each analysis, only one variable showed a correlation with the ratio of ARNT splice variant, and that was ER. Size, grade, node status, histology, and age were not related to ARNT splice-variant ratio. Splitting by median showed an inverse association with ER status ($P = 0.01$), confirmed on further analysis of four groups split by quartiles for ARNT ratio (Table 2). For example, only 2 of the 23 cases in the lowest quartile were ER negative compared with 11 of 21 in the highest quartile. As a continuous variable using Spearman rank, the correlation showed a negative coefficient, with ER of borderline significance ($P = 0.06$). Thus, each analysis showed: the higher the ratio, the more likely it was to be associated with ER-negative tumors.

**Relationship of ARNT Splice Variant to Relapse-free and OS.** Because the ratio of ARNT splice variant:full length may be vital for maximal biological affect, the relationship to prognosis was studied using the median as a cut point and the upper quartile and also the cases with ratios >10, i.e., a 1-log difference.

Analyzing by the median showed a lower survival for high-splice variants ratio, as did analysis of upper quartile
Although this was not significant. Analysis of the highest cases showed significantly worse RFS (P = 0.03; hazard ratio, 2.7; confidence interval, 1.1–6.7; Fig. 3B) and OS (Fig. 3C; P = 0.006; hazard ratio, 3.9; confidence interval, 1.5–10.2), using Kaplan-Meier statistics.

RFS and OS were analyzed by multivariate Cox analysis using the following factors: age, node status, size, ER, and EGFR as categorical variables. Lymph node status and ER were the factors with the Ps greater than 0.05 for OS, with hazard ratios of 3.3 and 0.4, respectively. ARNT splice-variant ratio was added into the model and showed that for RFS and OS, there were significantly independent effects associated with a worse prognosis (hazard ratio, 2.7; P = 0.03 and hazard ratio, 3.9; P = 0.006, respectively; Table 3) and the ARNT splice-variant ratio was the most significant factor.

DISCUSSION

This study shows for the first time that the ARNT splice variants, initially detected in ER-negative human breast cancer cell lines (13), exist in primary human breast cancers. Additionally, as predicted by the cell line results, there is a strong inverse relationship between the ARNT splice variant:full-length ratio and ER status.

The relevance of ARNT expression to hypoxia signaling was initially studied in cell lines, and we showed that inducibility of VEGF correlated with the pattern of splice variants. Although it may be expected that more aggressive tumors should have a more responsive hypoxia signaling pathway, the result shown with the human breast cell lines suggest that this is not the case for VEGF mRNA induction. Recent studies have reported that the induction of HIF-1α may increase apoptosis under hypoxia and also stabilize wild-type p53 (21, 22). In a cDNA array screen for genes induced by hypoxia in tumor cell lines, we found that several apoptotic genes were induced by HIF-1 (23). Therefore, reduction of the signaling pathway may be a mechanism of tumor progression under hypoxia.

We have studied VEGF inducibility by hypoxia in a larger panel of breast cell lines (24) and found that the lower inducibility of VEGF occurs for another hypoxia-regulated gene, lactate dehydrogenase. Also, the survival under hypoxia was greater for the same cells that had poorer VEGF induction. We and others have shown that MDA 435 and 231 grow better in vivo as xenografts than MDA 468, and T47D needs estrogen supplementation to grow in vivo. Overall, therefore, the expression of ARNT splice variants in these ER-negative cell lines is associated with a more aggressive phenotype, and the results imply that the splice variant is reducing hypoxia signaling.

Taking this hypothesis through to the clinical analysis, the first observation was the inverse association with ER status. Not all ER negative cases had the splice variant as the major form, as was also the case in the cell lines. None of the other tradi-

Table 2  Relation of ER status to ARNT splice variant ratio

<table>
<thead>
<tr>
<th>Ratio of ARNT splice variant:full-length ARNT</th>
<th>&lt;1.3</th>
<th>&lt;2.3</th>
<th>&lt;5.7</th>
<th>≥5.7</th>
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<tr>
<td>ER+</td>
<td>21</td>
<td>18</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>ER−</td>
<td>2</td>
<td>5</td>
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<td>25</td>
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</tr>
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</table>

Fig. 2  PCR results for analysis of splice variants of ARNT. RT-PCR analysis of ARNT mRNA transcripts from 16 of the breast cancer patients. Total RNA (300–1000 ng) was reverse transcribed and amplified using the ARNT forward and reverse primers as described in “Materials and Methods.” Arrows, wild-type (2.4 kb) and variant (1.1 kb) forms of ARNT cDNA.

Fig. 3  Survival curves of patients separated by ARNT splice variant ratio.
enzymes such as glutathione transferase (CYP 1A1, 1B1, and 1A2) but also Phase II metabolizing enzymes that can activate drugs including cytochrome P450s cytotoxic drugs (36, 37). The AhR signaling pathway regulates metabolizing enzymes response may confer resistance to certain


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