Close and Stable Relationship between Proliferation and a Hypoxia Metagene in Aromatase Inhibitor–Treated ER-Positive Breast Cancer

Zara Ghazoui1, Francesca M. Buffa2, Anita K. Dunbier1, Helen Anderson1, Tim Dexter1, Simone Detre2, Janine Salter1,2, Ian E. Smith2, Adrian L. Harris3, and Mitchell Dowsett1,2

Abstract

Purpose: The majority of breast cancer patients who have estrogen receptor positive (ER+) tumors whose proliferation is reduced after estrogen deprivation by aromatase inhibitors (AI). This study investigates any link between proliferation and hypoxia, a major determinant of tumor biology, and defines the effect of estrogen deprivation on hypoxia-associated genes.

Methods: Genome-wide expression profiles were obtained from tumor biopsies from 81 ER+ postmenopausal patients, before and after 2 weeks’ anastrozole treatment. A hypoxia metagene was developed by identifying genes clustered with classical hypoxia-regulated genes, excluding those associated with proliferation. Proliferation was measured by Ki67 and a proliferation metagene derived from two published breast cancer data sets.

Results: Hypoxia and proliferation metagenes were associated at baseline (Pearson correlation coefficient, \( r = 0.67, P < 10^{-4} \)) and after 2 weeks (\( r = 0.71, P < 10^{-4} \)). Hypoxia metagene at baseline was associated with 2-week Ki67 (\( r = 0.43, P = 0.0002 \)) and more weakly with poor 2-week Ki67 change consistent with a weak association with AI resistance. Hypoxia metagene was significantly downregulated with AI. This downregulation was significantly associated with change in the proliferation metagene and with Ki67 but, importantly, not with the substantial change in expression of classical estrogen-dependent genes.

Conclusions: Hypoxia metagene is closely associated with proliferation before and after AI treatment. The downregulation of hypoxia metagene after AI therapy is most likely the result of changes in proliferation. There may be a weak effect of hypoxia metagene on de novo resistance to AIs. These findings are important to consider in coapplication of antiproliferative agents with antiangiogenic or antihypoxia agents. Clin Cancer Res; 17(9): 3005–12. ©2011 AACR.

Introduction

At primary diagnosis, the majority of breast cancer patients have tumors that are estrogen receptor positive (ER+) and proliferate in response to estrogen. This knowledge has been exploited clinically by the development of endocrine agents such as tamoxifen, which compete with estrogen for the ER or aromatase inhibitors (AI), which block estrogen synthesis. Tamoxifen has been a standard endocrine therapy for many years; however, in postmenopausal women, the third generation AIs such as letrozole, anastrozole, and exemestane have been shown to be more effective than tamoxifen as adjuvant therapy (1). They are highly specific for the aromatase enzyme and achieve near complete inhibition of in vivo aromatization (2, 3). AIs are therefore excellent biological probes of the interaction between estrogen and ER+ breast cancer. Although proliferation as measured by expression of the nuclear antigen Ki67 is greatly reduced after treatment with AIs (4–6), their impact on other important facets of malignancy such as hypoxia is unknown.

Hypoxia is very common in breast cancer and occurs as a result of a reduction in the normal level of oxygen in a tissue due to an imbalance between oxygen delivery and oxygen consumption. Although hypoxia can lead to cancer cell death, if it is severe and prolonged, hypoxic cancer cells adapt to hypoxic conditions by undergoing genetic and adaptive changes. This adaptive process enables cell survival and even proliferation in a hypoxic environment (7). High proliferation may lead to a tumor outgrowing its vascular support and thereby generate hypoxia. Thus, changes in proliferation as achieved by AIs might substantially affect hypoxia. Application of agents targeted at
Translational Relevance

The majority of breast cancer patients have tumors that are estrogen receptor positive (ER+) and proliferate in response to estrogen. Hypoxia is a major determinant of the biology of solid malignancies and is a target for a number of therapeutic agents, including some that utilize hypoxia for drug activation. Proliferation is profoundly reduced in ER+ breast cancer after estrogen deprivation with an aromatase inhibitor (AI); however, little is known on the effects of AIs on hypoxia. Our data are the first to show the close relationship between a hypoxia metagene and proliferation in human breast cancer that is present at baseline and maintained during treatment with antiproliferative endocrine agents. These findings are important to consider in coapplication of antiproliferative agents such as AIs with antiangiogenic or antihypoxia agents.

hypoxia in association with such antiproliferative agents may be profoundly affected by this interaction.

An additional consideration for the combination of antiendocrine and hypoxia-directed agents is that the maintenance of a tumor in a hypoxic environment necessitates adaptive growth dependencies. Hypoxia may therefore lead to the production of growth factors that reduce an ER+ tumor dependence on estrogen as well as resistance to endocrine therapy. We have recently shown an effect of endocrine therapy (AI or tamoxifen) on stimulants of angiogenesis [vascular endothelial growth factor receptor (VEGFR) and VEGFR-I], which may have bearing upon this concept (8).

The aims of the current study were (i) to define the effect of estrogen deprivation by anastrozole on the expression of genes closely associated with hypoxia in ER+ breast cancer, (ii) to characterize any associations between changes in hypoxia and proliferation, and (iii) to assess whether hypoxia-regulated genes contribute to AI resistance.

Methods

Patient samples

Core-cut tumor biopsies were collected using 14-gauge needles from 81 postmenopausal women with stage I to IIIB ER+ early breast cancer before and after 2 weeks of anastrozole treatment in the anastrozole-only arm of a neoadjuvant trial (9). Further tumor biopsies were obtained from 20 of these patients after 16 weeks of anastrozole treatment prior to surgery. Clinical response was determined using the modified International Union Against Cancer/WHO assessment criteria (9). At each time point, one core cut was stored in RNA later at −80°C for RNA extraction and another fixed in neutral buffered formalin and paraffin embedded for immunohistochemistry. One 4-μm section from the surface of each core was stained with hematoxylin and eosin (H&E) to confirm the presence of cancerous tissue and histopathology. Total RNA was extracted using RNeasy (Qiagen) and RNA quality was assessed using an Agilent Bioanalyzer. Cores with 10% or more tumor were included on the basis that (a) the H&E slide could not be fully representative of the cellularity of the core and (b) clustering analysis, which was conducted as a quality assessment procedure showed that all apparently low cellularity specimens clustered either with their higher cellularity paired cores or other pairs of high cellularity. The median tumor percentage was 60% as measured by 2 independent pathologists. In addition, samples were analyzed only if their RNA integrity values were greater than 5.0. Eleven samples were excluded from this study because they failed the RNA integrity criterion.

Gene expression analysis and data processing

RNA amplification, labeling, and hybridization on HumanWG-6 v2 Expression BeadChips were carried out according to the manufacturer’s instructions (http://www.illumina.com). The HumanWG-6 v2 Expression BeadChip covers more than 48,000 transcript probes and its annotation is publicly available. Data were extracted by using BeadStudio (Illumina) software and were transformed and normalized using variance-stabilizing transformation and robust spline normalization method in the Lumi package in Bioconductor (http://www.bioconductor.org). Probes were discarded from further analyses if they were not detected in any of the samples (detection P > 1%).

Ki67 measurement

Immunohistochemical scoring for Ki67 was performed on 4-μm paraffin-embedded sections of tumor biopsies using the MIB1 antibody as previously described (10). Ki67 positive and negative cells were counted in 10 high-power fields (40× magnification), and the percentage change in Ki67 positive cells from baseline to 2 weeks posttreatment was then calculated.

Proliferation metagene development

Gene expression data from 2 breast cancer data sets (11, 12) were retrieved from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). Expression values were transformed using logarithms to base 2 and quantile or rank normalization was applied across all samples. A set of 4,391 probe sets was selected for further analyses after removing probes whose maximum levels of expression were below 7 and had an interquartile range below 0.5. A proliferation cluster was derived from each of the 2 breast cancer data sets independently, using flexible beta clustering with correlation distance, as the smallest cluster that contained 95% of the genes previously reported to be associated with proliferation (13–15). A proliferation metagene of 94 genes in our data set (Supplementary Table S1) was then derived by selecting the intersection of the 2 developed proliferation clusters. In each sample, the median expression levels for the genes in the proliferation metagene were calculated at baseline and after AI treatment. This proliferation metagene was validated in...
the current study by correlating its expression with Ki67 at baseline ($r = 0.62, P < 10^{-4}$).

**Hypoxia metagene development**

Gene expression data sets were retrieved from 5 large published breast cancer data sets (Table 1 and refs. 16–18). Data were filtered, normalized, and a hypoxia metagene was then derived by agglomerative clustering analysis as previously described (19). Genes were selected whose in vivo expression strongly associated with the expression of 10 well-characterized hypoxia-associated genes ($ADM, AK3L1, BNIP3, CA9, ENO1, HK2, LDHA, PGK1, SLC2A1, and VEGFA$) representing a wide range of known hypoxia-regulated pathways. For each gene, a metaconnectivity score was calculated; genes were then ranked using this score and a metagene was extracted as the top 100 ranked genes (19). Functional assessment using Gene Ontology (GO) Mining Tool was conducted to identify genes in the hypoxia metagene, which are directly involved in proliferation (Supplementary Table S2). Twenty-four genes that were found to be associated with proliferation according to their GO classification or were included in our proliferation metagene were removed before finalizing the hypoxia metagene (Supplementary Table S3). In each sample, the median expression levels of the genes in the finalized hypoxia metagene were computed prior to and post-AI treatment. Pearson correlations were conducted on the median expression values of the hypoxia and proliferation metagenes to identify any associations between them.

**Results**

**Hypoxia metagene**

The finalized breast cancer hypoxia metagene after subtraction of proliferation-associated genes is shown in Table 2. As expected this contained several genes that have been found to be induced by hypoxia (7, 20). These genes are involved in different pathways including glucose metabolism ($GAPDH$ and $ENO1$), angiogenesis ($VEGF$), and the regulation of apoptosis ($BNIP3$); these molecular pathways are in turn involved in a cellular response to hypoxia. To confirm the validity of the hypoxia metagene, we conducted a validation analysis of the genes in this metagene by using global gene expression data from published experiments in the MCF7 breast cancer cell line (21). Approximately 70% (47/70) of the genes in the hypoxia metagene were significantly regulated ($P < 0.05$) either by hypoxia or by HIF1α/HIF2α siRNAs (of which 26 in common; Supplementary Table S4). In addition, a literature search on the remaining 23 genes in the hypoxia metagene that were not significantly regulated by hypoxia in vitro revealed that 13/23 (60%) of these genes were linked to hypoxia in published literature (Supplementary Table S5). Thus the large majority of the genes in the hypoxia metagene are supported by experimental or previously published data.

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<td>315</td>
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**Table 2. Breast cancer hypoxia metagene excluding proliferation-associated genes**

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<tr>
<th>PGAM1</th>
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<th>ANKR3D7</th>
<th>ATP5G3</th>
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</tr>
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<td>TMEM70</td>
<td>TCEB1</td>
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<td>RANBP1</td>
<td>IMPAD1</td>
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</tr>
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<td>MRPL15</td>
<td>ATP1B3</td>
<td>IMPA2</td>
<td>SHMT2</td>
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</table>

NOTE: Genes are ordered by decreasing number of metaconnectivity score from PGAM1 (highest) to SHMT2 (lowest). The abbreviations are defined in Supplementary Table S3.
Expression of the hypoxia metagene in ER$^+$ breast cancer at baseline

Global gene transcription was used to measure the pretreatment expression levels of the hypoxia metagene, which was computed as the median log$_2$ expression of the genes in the hypoxia metagene in the 81 ER$^+$ breast cancer patients. Values were found to show an approximately 1.2 log range (Figs. 1A and 1B). The expression of the hypoxia metagene was associated with Ki67 (Pearson correlation coefficient, $r = 0.55$, $P < 10^{-4}$) and the expression of the proliferation metagene ($r = 0.67$, $P < 10^{-4}$) before AI treatment [Table 3 (A) and Figs. 1A and 1B]. AIs are known to suppress Ki67 radically in ER$^+$ breast cancer (4, 6); in this cohort of 81 patients, Ki67 was suppressed by a mean of 73.3% (SE = 3.3%) after 2 weeks of treatment. Pretreatment levels of the hypoxia metagene were associated with the change in Ki67 with a borderline statistical significance [Spearman rank correlation, $r_s = 0.22$, $P = 0.07$; Table 3(A)]. There was a poorer but still significant association of the pretreatment hypoxia metagene with 2-week Ki67 [as opposed to pretreatment Ki67; $r = 0.43$, $P = 0.0002$; Table 3(A)]. Incorporating the pretreatment expression levels of the hypoxia and the proliferation metagenes in a multivariate analysis to predict 2-week Ki67, the $P$ values were 0.11 and 0.05, respectively, showing a trend toward each metagene contributing independent information.

Data on clinical response were available on the patients who received anastrozole alone for their complete courses of neoadjuvant therapy ($n = 41$). Investigating these data, we found significantly higher levels of pretreatment expression levels of the hypoxia metagene in patients who showed stable disease or progression versus those who had complete or partial response [$n = 16$ vs. $25$, $P = 0.05$ ($t$-test)].

Effect of AI treatment on the hypoxia metagene

Because of the larger number of paired samples available to us at baseline and at 2 weeks, we have focused on the effects of 2 weeks of AI treatment (rather than 16 weeks). The hypoxia metagene was significantly downregulated over this period using Wilcoxon signed-rank test as shown in Figure 2 ($P < 10^{-4}$). Values were decreased by a mean of 9.8% (SE = 1%) This change in the hypoxia metagene correlated significantly with both the change in Ki67 ($r_s = 0.33$, $P = 0.007$) and change in the proliferation metagene ($r = 0.69$, $P < 10^{-4}$, Table 3(B); Figs. 3A and 3B).

In contrast, the change in the hypoxia metagene did not correlate with the substantial average change in 4 estrogen-dependent genes (TFF1/pS2, PDZK1, GREB1 and PGR) previously described as the AvERG (ref. 22; $r = 0.05$, $P = 0.7$) or in one of the most well characterized of these estrogen-dependent genes TFF1/pS2 [$r = 0.007$, $P = 0.9$; Table 3(B) and Supplementary Fig. S1).
Expression of the hypoxia metagene during AI treatment

The relationship of the hypoxia metagene with the proliferation metagene and with Ki67 that was seen prior to treatment was even stronger after 2 weeks of AI treatment but similar in the smaller number of samples available at 16 weeks. After 2 weeks of treatment, Pearson’s correlation coefficient for the hypoxia metagene with Ki67 and the proliferation metagene was 0.58 (\(P < 10^{-4}\)) and 0.71 (\(P < 10^{-4}\)), respectively, and after 16 weeks it was 0.50 (\(P = 0.03\)) and 0.73 (\(P = 0.0003\)), respectively (Table 3 (C) and 3(D); Figs. 1C and 1D). Notably, the correlations between the hypoxia and the proliferation metagenes were consistently stronger than those between the hypoxia metagene and Ki67. A possible explanation for this is that the Ki67 was measured on single section from paraffin-embedded cores, whereas the gene expression data for both of the metagenes were measured using different cores. In addition, the metagenes were both measured at the transcriptional level, whereas Ki67 was measured at the protein level.

External validation

To validate the finding that the expression of the hypoxia metagene was decreased after AI treatment in our data set, we have examined the effect of letrozole on the hypoxia metagene using publicly available global gene expression data of 58 patients before and after 2 weeks of treatment (23). In this data set, the hypoxia metagene was significantly downregulated (\(P = 0.04\)) using Wilcoxon test; the baseline expressions of the hypoxia and the proliferation metagenes were significantly correlated (\(r = 0.32, P = 0.01\)), and the association between changes in the expression of these 2 metagenes with AI treatment was also highly significant (\(r = 0.42, P = 0.001\)).

Discussion

The primary aim of the current study was to investigate the effects on hypoxia-associated genes by endocrine therapy using AIs in a neoadjuvant setting. Although the impact

<table>
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<th>Table 3. Correlations of the pretreatment, 2-week change, 2-week posttreatment, and 16-week posttreatment levels of the breast cancer hypoxia metagene</th>
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<tr>
<td>Pretreatment Ki67 (log2)</td>
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<tr>
<td>A. Pretreatment hypoxia metagene</td>
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<td>2-wk percentage change in Ki67</td>
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<td>r = 0.55, P &lt; 10^-4</td>
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<td>B. 2-wk change in hypoxia metagene</td>
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<td>2-wk Ki67 (log2)</td>
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<td>rs = 0.33, P = 0.007</td>
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<tr>
<td>C. 2-wk posttreatment hypoxia metagene</td>
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<tr>
<td>16-wk Ki67 (log2)</td>
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<tr>
<td>r = 0.58, P &lt; 10^-4</td>
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<tr>
<td>D. 16-wk posttreatment hypoxia metagene</td>
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</table>

Figure 2. Expression changes in the breast cancer hypoxia metagene in response to 2 weeks of AI treatment.
of AIs on proliferation as assessed by Ki67 has been previously established (4–6), the clinical effects of AIs on hypoxia have not been studied. In addition to evaluating those effects, our secondary aims were to identify any relationship between any changes in hypoxia and those in proliferation, and to assess whether hypoxia-associated genes contribute to AI resistance.

To address these aims, we studied global gene expression in 81 pre- and post–AI-treated ERþ breast cancers in postmenopausal women and found that treatment with anastrozole over 2 weeks resulted in significantly reduced expression levels of a breast cancer hypoxia metagene. Importantly, the change of expression of this metagene was associated with change in proliferation but not with the major change in classical estrogen-dependent genes also seen with AI treatment.

This observation suggests that the effect of estrogen deprivation on hypoxia-associated genes is more likely the result of changes in proliferation rather than removal of estrogenic stimuli from hypoxia-associated genes. The hypoxia metagene was also associated with proliferation at baseline and after 2 and 16 weeks of AI treatment, providing a consistent linkage between hypoxia and proliferation across all time points. Furthermore, the current data suggest that there may be a weak effect of the hypoxia metagene on de novo resistance to AIs based both on the 2 week change in Ki67 and clinical response, albeit on a relatively smaller set of patients for the latter parameter, but this is not as strong a relationship as that seen with many other factors related to endocrine resistance (24). Strength of the present study lies in the homogenous, well-annotated population as all the patients in our cohort were postmenopausal women with ERþ breast cancer from a single clinical trial. Furthermore, we had measurements from a sufficient number of primary untreated tumors and their matched post-treatment specimen to provide us with good statistical confidence.

A limitation is that although the genes in the hypoxia metagene are mostly well known to be associated with hypoxia and we subtracted genes that are directly associated with proliferation from the original metagene, a direct association with hypoxia itself has not been shown in this article, given that there is no gold standard method to measure hypoxia (25, 26). Also, a small number of genes with overlapping functions between proliferation and hypoxia, such as eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), which are not directly linked to proliferation by GO annotation remained in the hypoxia panel. Although the associations between the expression of the hypoxia metagene and proliferation in this study were highly significant, some of these correlations were relatively weak as might be expected in a clinical study with a heterogeneous population of tumors. Thus the interpretation of the results is necessarily cautious.

To determine whether the relationships between the hypoxia metagene and proliferation in this study are tumor-type specific, we have used a recently published combined hypoxia metagene developed using breast cancers as well as head and neck squamous cell cancers (19). The data showed that overall the associations between hypoxia and proliferation were stronger with the breast cancer specific hypoxia metagene than with the combined metagene, which implies some tumor specific differences in hypoxia responses (Supplementary Tables S6a and S6b).

Hypoxia is associated with poorer outcome (7) and a hypoxia metagene derived from head and neck cancers was found to be a prognostic marker for recurrence-free survival (27). More recent data also showed that a hypoxia metagene derived from multiple cancers including breast cancers was highly prognostic in independent data sets (19). In our study, a borderline relationship was found between the baseline expression values of the hypoxia metagene and both the change in Ki67 after 2 weeks of AI treatment and clinical response, suggesting that hypoxia may also be associated with resistance to AIs. This possibility, however, requires confirmation in larger cohorts of breast cancer patients.
The association of the hypoxia metagene and proliferation as assessed by Ki67 and gene expression at baseline persisted after 2 weeks and 16 weeks of treatment suggesting a strong link between these features even after a profound decrease in proliferation [mean fall of Ki67 = 73.3% (SE = 3.3%)] due to 2 weeks of estrogen deprivation. Although our data reveal highly significant correlations between the change in the hypoxia metagene and proliferation, some tumors with little change in the hypoxia metagene displayed the greatest decreases in proliferation (Fig. 3A) and other cancers with an increase in the hypoxia metagene had either a decrease or an increase in the proliferation metagene (Fig. 3A). Thus, although it may be important to consider these overall relationships in the rationale for combining antiendocrine with antihypoxia therapy, tailoring treatment in individual patients is confounded and impractical at this stage.

Given that the prognostic value of Ki67 has been found to be increased after 2 weeks of endocrine treatment in comparison with baseline values (28), this may also be the case with hypoxia. The current study design did not permit an assessment of this but is worthy of a future study in large presurgical trials such as the ongoing POETIC (Preoperative Endocrine Therapy for Individualizing Care) trial (Edura CT number: 2007-003877-21). The causes of tumor hypoxia are complex but involve a mismatch between oxygen delivery and consumption. Our data are consistent with proliferation, and the associated increased oxygen consumption, being a major cause of tumor hypoxia in breast cancer. This has implications for combination therapies with several classes of drugs. For instance, hypoxia-targeting agents such as bevacizumab are already under investigation with AI therapy. In this setting, our hypoxia metagene could potentially be used to guide decisions on whether or not bevacizumab should be combined with AI treatment on a patient-specific basis. In contrast, antiangiogenic agents, which should increase hypoxia, may be less effective in combination with endocrine therapy, which is reducing oxygen consumption. On the contrary, reduced oxygen consumption may increase intratumoral oxygen tension, as recently shown for AKT inhibition (29), which would be expected to enhance the effectiveness of radiation therapy. There are also many novel agents that target hypoxia in cancer, either requiring activation in hypoxia (30) or targeting hypoxia-regulated genes such as LDHA (31) and of course VEGF. In principle, they would be combined with other therapies that target breast cancer but just as with antiangiogenic agents, these may be less effective with endocrine therapy and careful consideration of trial design and patient selection will be needed in testing such approaches.

However, those patients with poorer response to AIs may still have proliferating hypoxic cells and therefore a dynamic approach to combinations based on early response to the endocrine therapy should be investigated.

In conclusion, this study has indicated a potential role for estrogen-driven proliferation in the generation of a tumor hypoxia metagene in ER+ breast cancer. These data should be taken into account when considering the coapplication of AIs with antiangiogenic or antihypoxia agents for breast cancer patients. There may also be a weak effect of the hypoxia metagene on de novo resistance to AIs but this remains to be further investigated in larger cohorts of breast cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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6. Ellis MJ, Tse Y, Young Q, White S, Proia AD, Murray J, et al. Estrone-independent proliferation is present in estrogen-receptor HER2-


Correction: Close and Stable Relationship between Proliferation and a Hypoxia Metagene in Aromatase Inhibitor–Treated ER-Positive Breast Cancer

In this article (Clin Cancer Res 2011;17:3005–12), which was published in the May 1, 2011 issue of Clinical Cancer Research (1), the accession numbers for the microarray data were not included. The microarray data have been submitted to ArrayExpress and the accession number is E-MTAB-520.

Reference

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