Hypoxia-Induced Gene Expression in Chemoradioresistant Cervical Cancer Revealed by Dynamic Contrast-Enhanced MRI

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Abstract
Knowledge of the molecular background of functional magnetic resonance (MR) images is required to fully exploit their potential in cancer management. We explored the prognostic impact of dynamic contrast-enhanced MR imaging (DCE-MRI) parameters in cervical cancer combined with global gene expression data to reveal their underlying molecular phenotype and construct a representative gene signature for the relevant parameter. On the basis of 78 patients with cervical cancer subjected to curative chemoradiotherapy, we identified the prognostic DCE-MRI parameter $A_{Brix}$ by pharmacokinetic analysis of pretreatment images based on the Brix model, in which tumors with low $A_{Brix}$ appeared to be most aggressive. Gene set analysis of 46 tumors with pairwise DCE-MRI and gene expression data showed a significant correlation between $A_{Brix}$ and the hypoxia gene sets, whereas gene sets related to other tumor phenotypes were not significant. Hypoxia gene sets specific for cervical cancer created in cell culture experiments, including both targets of the hypoxia inducible factor (HIF1α) and the unfolded protein response, were the most significant. In the remaining 32 tumors, low $A_{Brix}$ was associated with upregulation of HIF1α protein expression, as assessed by immunohistochemistry, consistent with increased hypoxia. On the basis of the hypoxia gene sets, a signature of 31 genes that were upregulated in tumors with low $A_{Brix}$ was constructed. This DCE-MRI hypoxia gene signature showed prognostic impact in an independent validation cohort of 109 patients. Our findings reveal the molecular basis of an aggressive hypoxic phenotype and suggest the use of DCE-MRI to noninvasively identify patients with hypoxia-related chemoradioresistance. Cancer Res; 72(20); 5285–95. ©2012 AACR.

Introduction
Magnetic resonance imaging (MRI) is an indispensable tool in cancer management, and is widely used for staging, treatment planning, and response monitoring (1). Functional MRI, such as dynamic contrast-enhanced (DCE)-MRI, provides biologic information related to tumor physiology (2). There is a growing interest to use dynamic contrast-enhanced MR imaging (DCE-MRI) to improve patient care by integrating anatomical and functional features (3). Locally advanced cervical cancer is one of the malignant diseases for which the inclusion of DCE-MRI could be helpful in clinical decision-making (1). Radiotherapy, often combined with cisplatin, is the primary treatment of choice and is challenging because of the risk of severe radiotoxicity in critical organs within the pelvis (4).

Studies have suggested that DCE-MRI of cervical cancer depicts functional features associated with progressive disease (5–8). The ability of the technique to visualize tumor aggressiveness has, however, hardly been used, partly because the biologic meaning of the images is not completely understood (9). DCE-MRI measures the temporal uptake pattern of a contrast agent in the tumor. When gadopentetate dimeglumine (Gd-DTPA) is used as contrast agent, the uptake mostly depends on the blood perfusion and the volume of the extracellular space (2). Biologic information can be extracted from the images by pharmacokinetic analysis of the uptake curves, where the Tofts and Brix models are most commonly applied (10). The Brix model has been recommended because the concentration of contrast agent in blood, that is, the arterial input function, is not needed in the calculations, making it particularly attractive for use in clinical routines (9). To fully exploit the potential of such models, a robust DCE-MRI biomarker should be identified and its molecular background clarified. Global gene expression profiles provide a detailed picture of the transcriptional program in tumors and thereby insight into the molecular phenotype. A few studies combining imaging and gene expression data have so far emerged, conveying results that encourage further work using this approach (11, 12).

In this study, we aimed to clarify the prognostic potential of the DCE-MRI Brix model parameters in patients with cervical cancer subjected to curative chemoradiotherapy and explore...
their molecular background by use of gene expression profiles. In a cohort of 78 patients, we carried out pharmacokinetic analyses of pretreatment DCE-MRI images and generated parameter histograms of each tumor to account for the intratumor heterogeneity in physiologic conditions. For the most prognostic parameter, the percentiles showing the strongest association with survival were used to extract a DCE-MRI parameter that was combined with global gene expression data. We further searched for a gene signature that reflected this parameter and contained molecular information about the potential aggressive phenotype visualized in the images. Our study shows the feasibility of this approach to decipher the molecular background of functional images and to identify a gene signature that reflects an aggressive cancer phenotype.

Materials and Methods

Patients and tumor specimens

A total of 137 patients with cervical carcinoma, prospectively recruited to our chemoradiotherapy protocol at the Norwegian Radium Hospital from 2001 to 2006, were included (Supplementary Table S1). Pretreatment DCE-MRI images suitable for analysis were available for 78 patients (DCE-MRI cohort). Three additional patients in our previous descriptive DCE-MRI study (7) were excluded because of unsatisfactorily fitting of the pharmacokinetic model. The remaining 109 patients served as an independent validation cohort based on their gene expression profiles. Possible pathologic lymph nodes in the pelvis at the time of diagnosis were evaluated by MRI according to the response evaluation criteria in solid tumors version 1.1 (13). All patients were treated with external radiation of 50 Gy to the tumor, parametria, and adjacent pelvic wall, and with 45 Gy to the remaining part of the pelvic region. This was followed by brachytherapy of 21 Gy to point A. Adjuvant cisplatin (40 mg/m²) was given weekly during the course of external radiation. Follow up consisted of clinical examinations, and when symptoms of relapse were seen, MR imaging of the pelvis and retroperitoneum as well as X-ray of thorax were carried out. Relapse (progressive disease) was classified as locoregional (regression within the irradiated field), distant, or both.

One to 4 tumor biopsies, approximately 5 × 5 × 5 mm in size, were taken before the start of therapy, immediately snap frozen, stored at −80 °C, and used for gene expression analysis. A separate specimen was fixed in 4% buffered formalin, paraffin-embedded, and used for immunohistochemistry. The study was approved by the regional committee of medical research ethics in southern Norway, and written informed consent was achieved from all patients.

DCE-MRI

A 1.5 T Signa Horizon LX tomography (GE Medical Systems) with a pelvic-phased array coil was used for MRI. Standard T1- and T2-weighted imaging was carried out in addition to DCE-MRI. To record the DCE-MRI series, an axial T1-weighted fast spoiled gradient recalled sequence was used (7). Gd-DTPA (0.1 mmol/kg body weight; Magnevist; Schering) was administered as a fast bolus injection. The sequence of DCE-MRI included 14 image series during a time period of 5 minutes, of which 1 series was recorded before the bolus injection and 13 after. The temporal resolution was 15 seconds for the first 11 image series and 1 minute for the remaining 2 series.

Image analysis

The tumor outline was assessed in axial T2-weighted images by an experienced radiologist, and transferred to the DCE series by coordinate mapping (Fig. 1A; ref. 7). The relative signal increase (RSI) was used as measure of the uptake of contrast agent and was calculated for each tumor voxel and time point, using the relation:

$$\text{RSI}(t) = \frac{S(t) - S(0)}{S(0)}$$

where $S(t)$ is the signal intensity at time $t$, and $S(0)$ is the signal intensity in the precontrast images. Using Levenberg–Marquardt least squares minimization (14), the Brix model (10) was fitted to the uptake curve of each tumor voxel, using the relation:

$$\text{RSI}(t) = A_{\text{Brix}} \cdot \frac{k_{ep}}{k_{el} - k_{ep}} \cdot \left( e^{-k_{el}t} - e^{-k_{ep}t} \right)$$

where $A_{\text{Brix}}$ is the amplitude, $k_{ep}$ the transfer rate of tracer from tissue to plasma, and $k_{el}$ the clearance rate of the tracer from plasma. The parameters were allowed to vary freely in the fitting, except for the constraints $A_{\text{Brix}} \cdot k_{ep} \cdot k_{el} \geq 0$. Parameter maps were created to visualize the distribution of parameter values within the tumors (Fig. 1B and data not shown). To investigate the correlation between the parameters and clinical outcome, a percentile screening method presented previously (7) was applied. In brief, for each tumor, a parameter histogram was generated and percentile values from the 1st to the 100th percentile were calculated (Fig. 1B and data not shown). On the basis of their nth percentile value, the patients were separated into 2 equally sized groups, and log-rank tests were employed to assess whether the risk of relapse differed significantly between the 2 groups. The relative risk (RR) and its 95% confidence interval (CI) were plotted as a function of percentile, and for $A_{\text{Brix}}$, the prognostic parameter was calculated as the mean value over the percentile interval with the most significant RR; that is, with the strongest association to outcome (Fig. 1C). For $k_{tp}$ and $k_{el}$, the associations with survival were nonsignificant or significant only within a small percentile interval (Supplementary Fig. S1), and they were therefore not included in further analyses.

Cell lines and hypoxia treatment

The HeLa, SiHa, and CaSkii cervical cancer cell lines from American Type Culture Collection were used to generate cervical cancer–specific gene sets of hypoxia responsive genes. The cells were identity tested before the experiments by short tandem repeat (STR) profiling using Powerplex 16 (Promega), which amplifies 15 STR loci and amelogenin for gender identification: Penta E, D18S51, D21S11, TH01, D3S1358, F GA,
TPOX, D8S1179, vWA, Amelogenin, Penta D, CSF1PO, D16S539, D7S820, D13S317, and D5S818.

The cells were incubated in Dulbecco’s modified Eagle medium with GlutaMAX supplemented with 10% fetal calf serum and 100 U/mL penicillin/streptomycin (Gibco) under a 5% CO2 atmosphere at 37°C. Cells were plated out in plastic dishes (3 x 10^5 cells for 6 cm dishes, 9 x 10^5 for 10 cm dishes) 24 hours before exposure to hypoxic (0.2% O2, 5% CO2) or normoxic (95% air, 5% CO2) conditions for 24 hours at 37°C. The hypoxia treatment was carried out in an Invivo2200 chamber (Ruskinn Technology Ltd.) with accurate O2 and CO2 controls. The treatment conditions of 24 hours with 0.2% O2 were selected to mimic conditions with prolonged hypoxia and ensure a response by HIF1α and possibly by the unfolded protein response (UPR; ref. 15).

The cell-cycle distribution was analyzed on an LSR II flow cytometer (Becton Dickinson) after fixation in 70% ethanol and staining with 1.5 μg/mL Hoechst 33258. Western blots were carried out by lysing cells with 10 mmol/L Tris HCl lysis buffer (pH 7.5) containing 2% SDS and 100 μmol/L Na3VO4. The proteins were separated by 8% Tris-HEPES-SDS polyacrylamide gels (Pierce Biotechnology), blotted on a PVDF membrane and stained with the monoclonal mouse HIF1α antibody clone 54 (1:800, no. 610958; BD Transduction Laboratories) and donkey antimouse secondary antibody (715-001-003; Jackson ImmunoResearch Laboratories, Inc.).

Gene expression analysis
Gene expression profiling of 155 patients (46 in the DCE-MRI cohort, 109 in the validation cohort) as well as normoxia- and hypoxia-treated cell lines was carried out using the Illumina bead arrays human WG-6 v3 (Illumina Inc.) with 48803 transcripts (16). In brief, total RNA was isolated from the frozen tumor specimens using Trizol reagent (Invitrogen) and from the cell lines using RNeasy MiniKit (Qiagen). cRNA was synthesized, labeled, and hybridized to the arrays. Signal extraction and quantile normalization were carried out by the software provided by the manufacturer (Illumina Inc.).
Log-transformed data were used in the analyses. All clinical specimens had more than 50% (median 70%) tumor cells in hematoxilin and eosin stained sections derived from the central part of the biopsy. RNA from different biopsies of the same tumor was pooled. The Illumina data have been deposited to the GEO repository (GSE36562).

Computational analysis of gene expression profiles

To explore biologic processes associated with $A_{\text{Brix}}$, a list of genes was generated from the expression profiles of 46 DCE-MRI patients, based on the Spearman rank correlation between gene expression and $A_{\text{Brix}}$. A cut off $P$-value of 0.05 was used to achieve an appropriate number of genes for further analysis. The gene ontology (GO) categories of the correlating genes were compared with those of all genes on the array using the master-target procedure with the Fisher’s exact test in the eGOn software, where a multiple testing algorithm was used to control the false discovery rate (FDR; ref. 17). Furthermore, gene set analysis tool Significance Analysis of Microarrays (SAM-GS), which is based on the moderated t-statistic in SAM (18) and with the same multiple testing procedure as above to control FDR, was used for a supervised statistic in SAM (18) and with the same multiple testing algorithm was used to control the false discovery rate (FDR; ref. 17). Furthermore, the gene set analysis tool Significance Analysis of Microarrays for Gene Sets (SAM-GS), which is based on the moderated $t$-statistic in SAM (18) and with the same multiple testing procedure as above to control FDR, was used for a supervised statistic in SAM (18) and with the same multiple testing algorithm was used to control the false discovery rate (FDR; ref. 17).

Table 1. Gene sets associated with $A_{\text{Brix}}$

<table>
<thead>
<tr>
<th>Gene set</th>
<th>No. of genes</th>
<th>$P$</th>
<th>Adj. $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia cervical up $\times 3$</td>
<td>79</td>
<td>0.009</td>
<td>0.122</td>
</tr>
<tr>
<td>Hypoxia cervical up and literature</td>
<td>286</td>
<td>0.018</td>
<td>0.122</td>
</tr>
<tr>
<td>MENSE_HYPOXIA_UP ($22^a$)</td>
<td>95</td>
<td>0.025</td>
<td>0.122</td>
</tr>
<tr>
<td>WINTER_HYPOXIA_UP ($24^a$)</td>
<td>91</td>
<td>0.032</td>
<td>0.122</td>
</tr>
<tr>
<td>REACTOME_CELL_CYCLE_CHECKPOINTS</td>
<td>110</td>
<td>0.038</td>
<td>0.122</td>
</tr>
<tr>
<td>KEGG_NON_HOMOLOGOUS_END_JOINING</td>
<td>13</td>
<td>0.052</td>
<td>0.138</td>
</tr>
<tr>
<td>Hypoxia cervical down and literature</td>
<td>183</td>
<td>0.068</td>
<td>0.156</td>
</tr>
<tr>
<td>KEGG_HOMOLOGOUS_RECOMBINATION</td>
<td>27</td>
<td>0.082</td>
<td>0.163</td>
</tr>
<tr>
<td>STARMANS_WOUND_SIGNATURE (23)</td>
<td>413</td>
<td>0.093</td>
<td>0.166</td>
</tr>
<tr>
<td>CHIANG_LIVER_CANCER_SUBCLASS_PROLIFERATION_UP ($20^a$)</td>
<td>136</td>
<td>0.127</td>
<td>0.202</td>
</tr>
<tr>
<td>STARMANS_PROLIFERATION_SIGNATURE (23)</td>
<td>104</td>
<td>0.172</td>
<td>0.239</td>
</tr>
<tr>
<td>Hypoxia cervical down $\times 3$</td>
<td>10</td>
<td>0.179</td>
<td>0.239</td>
</tr>
<tr>
<td>KEGG_PROTEIN_PROCESSING_IN_ER</td>
<td>162</td>
<td>0.258</td>
<td>0.296</td>
</tr>
<tr>
<td>ISHIKAMI_RADIATION_RESISTANCE (21)</td>
<td>25</td>
<td>0.259</td>
<td>0.296</td>
</tr>
<tr>
<td>KEGG_BASE_EXCISION_REPAIR</td>
<td>32</td>
<td>0.527</td>
<td>0.562</td>
</tr>
<tr>
<td>AMUNDSON_GAMMA_RADIATION_RESISTANCE (19)$^c$</td>
<td>17</td>
<td>0.585</td>
<td>0.585</td>
</tr>
</tbody>
</table>

NOTE: Gene expression profiles of tumors with high (above median) and low (below median) $A_{\text{Brix}}$ were compared.

$^a$Gene sets in bold were created from cervical cancer cell lines in the present work, whereas the remaining gene sets were taken from the literature or from the Reactome, KEGG, or Molecular Signatures databases. References to the relevant literature are listed.

$^b$The $P$ values were adjusted for multiple testing.

$^c$Obtained from the Molecular Signatures Database (MSigDB) v3.0.

Immunohistochemistry

Immunohistochemistry (IHC) was carried out on 32 selected tumors with the monoclonal mouse HIF1α antibody clone 54 (1:25, no. 610958; BD Transduction Laboratories), binding the amino acid 610 to 727 on the HIF1α protein. The tissue sections (4 μm) were stained using the Dako EnVisionTM Flex+ System (K8012; Dako). For antigen retrieval, the PT-Link (Dako) and EnVision Flex target retrieval solution at a high pH were used, and the sections were incubated with the HIF1α antibody for 30 minutes. A cervical tumor known to express HIF1α was used as a positive control, whereas as a negative control the antibody was substituted with mouse myeloma protein of the same concentration and subclass as the HIF1α antibody. Nuclear staining was scored on the basis of the percentage of positive tumor cells: 0, 0%; 1, 1% to 10%; 2, 11% to 25%; 3, 26% to 50%; 4, 51% to 75%; and 5, >75%. The cytoplasmic staining was generally weak or absent, and was therefore not quantified. The scoring was carried out by an experienced scientist at the Department of Pathology (R.H.) who was blinded to the DCE-MRI data.
Figure 2. Association between $A_{\text{Brix}}$ and HIF1α protein expression. A, tumor $A_{\text{Brix}}$ maps superimposed on T2-weighted MR images of 2 different patients with cervical cancer. Purple color represents $A_{\text{Brix}}$ values of 0 and red represents values of ≥5.0. B, representative sections showing HIF1α expression of the tumors in A. Inset shows nuclear HIF1α expression. Original magnification, ×4; inset image, ×10. A and B, a tumor with low (below median, upper) and high (above median, lower) $A_{\text{Brix}}$, as calculated from the tumor histogram as the mean value over the 20th to 30th percentiles. C, box plot (median, first, and third quartile) showing nuclear HIF1α protein expression in tumors with low ($n = 17$) and high ($n = 15$) $A_{\text{Brix}}$, respectively. The whisks extend to the farthest points that are not outliers. The patients are grouped on the basis of the median $A_{\text{Brix}}$ in the 78 DCE-MRI patients. *, $P < 0.01$ (Mann–Whitney U test).

Statistics

Mann–Whitney U test was used for comparison of the protein expression level of HIF1α in the patient group with low $A_{\text{Brix}}$ versus high $A_{\text{Brix}}$. In the survival analyses, the endpoint was progression-free survival, where the time from diagnosis to cancer-related death or to the first event of relapse was used, or locoregional control, which was defined as control within the irradiated pelvic region including lymph nodes. Thirteen patients died of causes not related to cancer and were censored. Cox proportional hazard analysis was used to evaluate the prognostic value of various parameters with respect to progression-free survival and locoregional control. Kaplan-Meier curves were compared using log-rank test. P-values <0.05 were considered significant.

Results

Patients with low $A_{\text{Brix}}$ have a poor outcome after chemoradiotherapy

The prognostic potential of the DCE-MRI $A_{\text{Brix}}$ parameter was explored by evaluating the relative risk of all percentiles in the $A_{\text{Brix}}$ histograms, which enabled us to judge different parts of the histograms separately and identify the one with the strongest relationship to outcome (7). The lower $A_{\text{Brix}}$ values harbored the strongest prognostic information, and a broad range from the 6th to 59th percentile was associated with progression-free survival at a significance level below 0.08 (Fig. 1C), indicating considerable robustness in the $A_{\text{Brix}}$ parameter (7). The interval from the 8th to 36th percentile had a significance level below 0.05, whereas the 20th to 30th percentiles were the most significant ones ($RR < 0.44$, $P < 0.018$; Fig. 1C). The mean of $A_{\text{Brix}}$ values over the 20th to 30th percentiles was therefore used as the representative $A_{\text{Brix}}$ imaging parameter. This $A_{\text{Brix}}$ parameter varied considerably among the patients, ranging from 0.59 to 3.21, with a median of 1.50. Patients with an $A_{\text{Brix}}$ below the median showed a poor outcome compared with the others, and the probability of survival differed with more than 30% at 5 years follow-up between the 2 groups ($P = 0.004$; Fig. 1D).

Low $A_{\text{Brix}}$ associates with upregulation of hypoxia response genes and HIF1α protein

To examine whether the differences in $A_{\text{Brix}}$ among the tumors were reflected in the transcriptional program of specific biologic processes, we combined the gene expression profiles of 46 tumors with the DCE-MRI data. We first used an unsupervised GO analysis to obtain a general picture of the biology in low $A_{\text{Brix}}$ tumors, where the genes with the highest correlation between expression and $A_{\text{Brix}}$ were included. Four major significant processes were identified: metabolism, cell cycle, cellular component organization and biosynthesis, and response to DNA damage stimulus (Supplementary Table S2). On the basis of these results, a supervised gene set analysis was carried out on all genes on the array to more specifically investigate phenotypes that could be reflected in $A_{\text{Brix}}$. The correlation between $A_{\text{Brix}}$ and the categories metabolism, cell cycle, and cellular component organization and biosynthesis could indicate both altered proliferation and presence of hypoxia. Published gene sets associated with these phenotypes were therefore included (20, 22–24). To cover the process response to DNA damage stimulus, published gene sets representing intrinsic radioresistance were used (19, 21) as well as the KEGG DNA repair pathways for nonhomologous end-
joining, homologous recombination, and base excision repair. The cell-cycle checkpoints pathway from Reactome was also included to represent both this process and the cell-cycle category. Moreover, the KEGG pathway for protein processing in endoplasmic reticulum (ER) was used to cover the metabolism category more thoroughly because the protein modification process was one of its significant subcategories. Finally, we included a published gene set associated with wound healing that has shown prognostic impact in several other tumor types (23).

The most significantly differentially expressed gene sets when comparing tumors with high (above median) and low (below median) $A_{Brix}$ were those representing hypoxia ($P = 0.025$ and 0.032; Supplementary Table S3). The cell-cycle checkpoints pathway was also significant ($P = 0.038$), and a closer look at its genes revealed that several of those that were correlated with $A_{Brix}$ are known to be activated under hypoxia, such as ATR and CHEK2 (25). Thus, $A_{Brix}$ seemed to be associated with the transcriptional program regulated under hypoxia.

The transcriptional hypoxia response has been shown to differ among tumor types (26). A cervical cancer–specific hypoxia gene set could possibly be more strongly associated with $A_{Brix}$ than the gene sets used above and thus contain genes

### Table 2. The DCE-MRI hypoxia gene signature

<table>
<thead>
<tr>
<th>Probe ID*</th>
<th>Gene symbolb,c</th>
<th>Gene name</th>
<th>$P^d$</th>
<th>Corr$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2470341</td>
<td>ALDOA</td>
<td>Aldolase A, fructose-bisphosphate</td>
<td>0.038</td>
<td>0.307</td>
</tr>
<tr>
<td>1990491</td>
<td>AK2</td>
<td>Adenylate kinase 2</td>
<td>0.038</td>
<td>0.306</td>
</tr>
<tr>
<td>160148</td>
<td>AK3L1</td>
<td>Adenylate kinase 4</td>
<td>0.010</td>
<td>0.378</td>
</tr>
<tr>
<td>2640386</td>
<td>B3GNT4</td>
<td>UDP-GlcNAc:betaGal beta-1,3-N-acetylgalcosaminyltransferase 4</td>
<td>0.004</td>
<td>0.416</td>
</tr>
<tr>
<td>1170338</td>
<td>SCARB1</td>
<td>Scavenger receptor class B, member 1</td>
<td>0.003</td>
<td>0.314</td>
</tr>
<tr>
<td>4640041</td>
<td>CLK3</td>
<td>CDC-like kinase 3</td>
<td>0.046</td>
<td>0.295</td>
</tr>
<tr>
<td>7380634</td>
<td>C2ORF20</td>
<td>Chromosome 20 open reading frame 20</td>
<td>0.017</td>
<td>0.350</td>
</tr>
<tr>
<td>630674</td>
<td>ECE2</td>
<td>Endothelin converting enzyme 2</td>
<td>0.017</td>
<td>0.350</td>
</tr>
<tr>
<td>4780671</td>
<td>ERO1L</td>
<td>ERO1-like (S. cerevisiae)</td>
<td>0.019</td>
<td>0.343</td>
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<tr>
<td>2640048</td>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>0.041</td>
<td>0.302</td>
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<tr>
<td>6660601</td>
<td>HMOX1</td>
<td>Heme oxygenase (decycling) 1</td>
<td>0.024</td>
<td>0.333</td>
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<tr>
<td>2100196</td>
<td>ISG15</td>
<td>ISG15 ubiquitin-like modifier</td>
<td>0.038</td>
<td>0.308</td>
</tr>
<tr>
<td>7400653</td>
<td>PFKFB4</td>
<td>6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4</td>
<td>0.035</td>
<td>0.311</td>
</tr>
<tr>
<td>270408</td>
<td>P4HA2</td>
<td>Prolyl 4-hydroxylase, alpha polypeptide II</td>
<td>0.050</td>
<td>0.291</td>
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<tr>
<td>2760427</td>
<td>PYGL</td>
<td>Phosphorylase, glycogen, liver</td>
<td>0.011</td>
<td>0.371</td>
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<tr>
<td>6280632</td>
<td>RPL36A</td>
<td>Ribosomal protein L36a</td>
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<td>0.302</td>
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<td>2190408</td>
<td>UPK1A</td>
<td>Uroplakin 1A</td>
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<td>830619</td>
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<td>DNA-damage-inducible transcript 3</td>
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<tr>
<td>2760008</td>
<td>KCTD11</td>
<td>Potassium channel tetramerisation domain containing 11</td>
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<td>780524</td>
<td>PVR</td>
<td>Poliovirus receptor</td>
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<tr>
<td>4390619</td>
<td>RHOC</td>
<td>Ras homolog gene family, member C</td>
<td>0.043</td>
<td>0.299</td>
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<tr>
<td>1170170</td>
<td>STC2</td>
<td>Stanniocalcin 2</td>
<td>0.007</td>
<td>0.390</td>
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<td>2100341</td>
<td>C14orf2</td>
<td>Chromosome 14 open reading frame 2</td>
<td>0.030</td>
<td>0.321</td>
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<td>Chromosome 19 open reading frame 53</td>
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<td>1980369</td>
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<td>5900025</td>
<td>FGFI1</td>
<td>Fibroblast growth factor 11</td>
<td>0.033</td>
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<td>1300603</td>
<td>SH3GL3</td>
<td>SH3-domain GRB2-like 3</td>
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<td>0.307</td>
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<td>5690431</td>
<td>SNTA1</td>
<td>Syntrophin, alpha 1 (dystrophin-associated protein A1, 59 kDa, acidic component)</td>
<td>0.012</td>
<td>0.366</td>
</tr>
<tr>
<td>3440070</td>
<td>SPAG7</td>
<td>Sperm associated antigen 7</td>
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<td>0.372</td>
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<tr>
<td>2970017</td>
<td>S100A2</td>
<td>S100 calcium binding protein A2</td>
<td>0.009</td>
<td>0.381</td>
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<td>3420671</td>
<td>TRAPPC1</td>
<td>Trafficking protein particle complex 1</td>
<td>0.008</td>
<td>0.384</td>
</tr>
</tbody>
</table>

*illumina probe ID.

*bHUGO gene symbol.

cGenes in bold are known HIF1α targets and underlined genes are known to be involved in the unfolded protein response.

dCorrelation coefficient (Corr) and $P$ value in Spearman rank correlation analysis of $A_{Brix}$ versus gene expression.
that would be important for the creation of a robust gene signature that reflected $A_{Brix}$. We therefore generated hypoxia gene sets based on the expression profiles of cervical cancer cells grown under hypoxia. HIF1α protein was upregulated in all 3 cell lines by the hypoxia treatment, whereas only minor changes were observed in the cell-cycle distributions (Supplementary Fig. S2A and S2B). Four different gene sets were generated from the hypoxia induced expression changes; genes upregulated in all 3 cell lines, genes upregulated in one of the cell lines and confirmed as hypoxia regulated by the literature, and correspondingly for the downregulated genes (Supplementary Methods S1 and S2).

In the second round of gene set analysis, the cervical cancer–specific hypoxia gene sets with upregulated genes were found to be the most significant ones (Table 1). These results supported the suggestion of a correlation between $A_{Brix}$ and hypoxia, and showed that the cervical cancer–specific gene sets could be useful in the construction of an $A_{Brix}$ gene signature.

To confirm the above finding of a correlation between $A_{Brix}$ and hypoxia, protein expression of HIF1α was assessed by immunohistochemistry in the 32 remaining DCE-MRI patients who had not been evaluated in the gene expression study. HIF1α was selected as hypoxia marker because a hypoxia-induced HIF1α response was observed in the cell lines (Supplementary Fig. S2B). The patients with low $A_{Brix}$ had a significantly higher HIF1α expression compared with those with high $A_{Brix}$ ($P = 0.004$; Fig. 2A–C). In accordance with this result, several of the HIF1α targets were upregulated in tumors with low $A_{Brix}$ in the DCE-MRI patients used for gene expression analysis. We thus concluded that low $A_{Brix}$ was associated with tumor hypoxia and upregulation of hypoxia response genes.

$A_{Brix}$ reflects a hypoxia gene signature with prognostic impact in an independent cohort

To construct a DCE-MRI signature with the most important genes reflected by $A_{Brix}$, we selected the 4 significant hypoxia
gene sets (Table 1), and extracted the 31 genes with a negative correlation between the gene expression and \(A_{Brix}\); that is, the genes that were upregulated in tumors with low \(A_{Brix}\). Most of these 31 genes are known to participate in biologic processes affected by hypoxia, such as energy metabolism, cell cycle, and proliferation, including HIF1\(\alpha\) targets such as \(ALDOA\), \(STC2\), and \(HMOX1\) (27–29), and genes involved in the UPR (\(DDIT3\), \(STC2\), \(ERO1L\), and \(AK2\); refs. 30–32; Table 2), suggesting that several parts of the hypoxia induced transcriptional program were reflected by \(A_{Brix}\).

To ensure that the signature sufficiently represented the \(A_{Brix}\) parameter, we carried out unsupervised clustering of the 46 DCE-MRI patients based on the expressions of the 31 genes. Clustering showed 2 groups with different outcome, for which the one with high expression of hypoxia responsive genes had low \(A_{Brix}\) (\(P<0.001\)) and poor outcome (\(P=0.011\)) as compared with the other (Fig. 3A). We further calculated a hypoxia score for each tumor by averaging the median centered expression levels for the 31 genes, as described (26). In line with the above results, patients with a high hypoxia score had low \(A_{Brix}\) (\(P<0.001\)) and poor outcome (\(P=0.011\)) compared with the others (Fig. 3B). The DCE-MRI hypoxia gene signature therefore seemed to satisfactorily represent the \(A_{Brix}\) parameter, including its relationship to clinical outcome.

The prognostic impact of the gene signature was further validated in an independent cohort of 109 patients with cervical cancer. In this validation set, the patients who clustered together because of high expression of the genes in the signature had a significantly worse outcome than the remaining patients (\(P=0.002\); Fig. 4A). When assessing the hypoxia score in this cohort, the patients with a high score had a poor outcome compared with those with a low score (\(P=0.006\) Fig. 4B). The prognostic value of the signature was therefore confirmed. To assess the importance of the signature in comparison with existing clinical markers, the patients from the DCE-MRI cohort and the validation cohort were merged, and a multivariate Cox analysis was carried out on the resulting group of 155 patients. The hypoxia score emerged as a prognostic factor independent of lymph node status, FIGO stage, and tumor volume for both progression-free survival and locoregional control (Table 3).

**Discussion**

By integrating DCE-MRI and global gene expression data of cervical cancers, we found that the images reflect a transcriptional program regulated under hypoxia. This program included a gene signature with prognostic impact in an independent validation cohort, thus pointing to hypoxia-regulated
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Table 3. Cox regression analysis of the hypoxia score and clinical variables

<table>
<thead>
<tr>
<th>Factor</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>Relative Risk</td>
</tr>
<tr>
<td>Progression-free survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node status</td>
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<td>2.20</td>
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<tr>
<td>Tumor volume&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>3.33</td>
</tr>
<tr>
<td>FIGO stage&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>3.40</td>
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<tr>
<td>Hypoxia score&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.001</td>
<td>2.90</td>
</tr>
<tr>
<td>Locoregional control</td>
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<td></td>
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<tr>
<td>Lymph node status</td>
<td>0.120</td>
<td>2.27</td>
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<tr>
<td>Tumor volume&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.102</td>
<td>2.60</td>
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<tr>
<td>FIGO stage&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.080</td>
<td>2.48</td>
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<tr>
<td>Hypoxia score&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.033</td>
<td>3.48</td>
</tr>
</tbody>
</table>

NOTE: The analysis was carried out on 155 patients, including both the DCE-MRI cohort and the validation cohort.

Abbreviations: FIGO, Federation International de Gynecologie et d’Obstetrique; N.S., nonsignificant.

<sup>a</sup>Tumor size was divided into 2 groups on the basis of the median volume of 43.8 cm<sup>3</sup>.

<sup>b</sup>FIGO stage was divided into 2 groups: 1b–2b and 3a–4a.

<sup>c</sup>The hypoxia score was divided into 2 groups, based on values <0 and >0.

pathways that may promote cervical cancer aggressiveness. Tumor hypoxia is a known prognostic factor in many types of cancer, including cervical cancer (33), and would therefore be valuable to implement in clinical decision-making. Our work is the first to provide molecular insight into the DCE-MRI images that could facilitate this strategy, encouraging the use of DCE-MRI as a tool to handle hypoxia-induced chemoradioresistance in cervical cancer.

Several evidences for a relationship between low A<sub>Brix</sub> and tumor hypoxia were provided by our work. The hypoxia gene sets were significantly associated with A<sub>Brix</sub>, and revealed upregulation of hypoxia response genes including HIF1α targets in tumors with low A<sub>Brix</sub>. In line with this, tumors with low A<sub>Brix</sub> had higher protein expression of HIF1α compared with those with high A<sub>Brix</sub>. The results from the GO analysis were also in accordance with these conclusions, as the biologic processes metabolism, cell cycle, and DNA damage signaling, which were associated with A<sub>Brix</sub>, are important targets for the hypoxia response (25, 28, 34). In particular, genes from the metabolism category encoding the glycolytic enzymes ALDOA, GAPDH, and PFKFB4 were found to be upregulated in tumors with low A<sub>Brix</sub>, consistent with the increased glycolytic activity often seen in hypoxic tumors (28). Loncaster and colleagues (5) showed a relationship between A<sub>Brix</sub> and oxygen tension in cervical tumors, as measured by Eppendorf pO<sub>2</sub> histogram, supporting our conclusion.

Extraction of genes from the hypoxia gene sets that were upregulated in tumors with low A<sub>Brix</sub> resulted in a DCE-MRI hypoxia gene signature that was associated with the clinical outcome, independent of clinical markers for progression-free survival. This suggests that the gene signature associated with A<sub>Brix</sub> provides information of disease progression that is not covered by the conventional clinical parameters. In addition, the gene signature appeared as the most significant factor for locoregional control, indicating that hypoxia as measured by A<sub>Brix</sub> could be a well-needed biomarker of local recurrence in cervical cancer. Furthermore, while previous studies have demonstrated the importance of hypoxia in resistance to radiotherapy (33), the strong prognostic impact of this hypoxia gene signature indicates that hypoxia also influences the effect of chemoradiotherapy in cervical cancer patients.

The use of global gene expression data enabled detailed insight into the transcriptional program reflected by A<sub>Brix</sub>. The response to hypoxia occurs through different pathways, and may involve activation of HIFs, the UPR or ER stress response, and signaling through the mechanistic target of rapamycin (mTOR) kinase (32). Eight of the 31 genes in the DCE-MRI hypoxia gene signature are known HIF1α targets, namely ALDOA, ERO1L, GAPDH, PFKFB4, PHHA2, C4orf3, HOX1, and STC2 (27, 29, 32, 35, 36), although SCARB1 is a target of HIF2 (the endothelial PAS domain protein 1, EPAS1; ref. 36). The protein encoded by one of the other genes, RHOC, is indirectly associated with HIF1α through its interaction with the von Hippel-Lindau tumor suppressor (VHL; ref. 37), which degrades HIF1α. In addition, AK2, ERO1L, DDIT3 (also known as CHOP), and STC2 have been found to be involved in the UPR (30–32, 38). It thus seems that processes associated with both HIF activation and UPR may be important in the hypoxic phenotype depicted by low A<sub>Brix</sub>. The involvement of HIF activation was further supported by the high HIF1α protein expression in these tumors.

Current knowledge of how these genes influence cervical cancer progression is sparse. However, several of them or their encoded proteins, including STC2, PFKFB4, RHOC, S100A2, HMOX1, and ISG15, have been associated with poor prognosis in other cancer types (39–44). Moreover, upregulation of ALDOA, GAPDH, and PFKFB4 suggest a high glycolytic activity, as mentioned above, which has been associated with aggressiveness (28). Furthermore, SCARB1, PVR, and RPL36A have been shown to promote rapid proliferation (45–47), and...
AK4 may play a critical role in both cellular survival and proliferation during stress conditions such as hypoxia (48). Both DDIT3 and STC2 are targets of the activating transcription factor ATF4, which is induced by the eukaryotic translation initiation factor kinase EIF2AK3 (PERK) in response to hypoxia (31, 32, 49). STC2 has been shown to inhibit apoptosis and to induce proliferation and invasiveness in response to HIF1α or ER stress during hypoxia (27, 31, 50). DDIT3 on the other hand, is a proapoptotic protein, but was recently shown to protect tumor cells under hypoxia through the regulation of autophagy, proposing a role in the balance between autophagy and apoptosis (49). It may thus be speculated that the combined action of these 2 genes in tumors with low $A_{\text{Brix}}$ promotes the adaption to hypoxia by inhibiting apoptosis and inducing autophagy. Taken together, these studies support a role of the signature genes in the development of an aggressive hypoxic phenotype.

Using DCE-MRI to assess hypoxia associated aggressiveness in cervical cancer is appealing because MRI is already in routine use for patient diagnosis at many hospitals (1). Furthermore, the use of noninvasive imaging to assess the molecular hypoxia phenotype could potentially reduce the need of invasive biopsy procedures and be carried out during the course of treatment for response evaluation. $A_{\text{Brix}}$ seems to be a valuable DCE-MRI parameter for this purpose because of its strong correlation to outcome in our study. The robustness of the parameter, as suggested by the percentile analysis of $A_{\text{Brix}}$ histograms, is supported by the cervical cancer study of Loncaster et al. (5), where $A_{\text{Brix}}$ also had prognostic impact although the patient characteristics and MRI protocol differed from ours. In the present work, we have showed how noninvasive imaging by means of DCE-MRI and $A_{\text{Brix}}$ assessment may visualize hypoxia and its molecular basis in chemoradioreistant tumors. Our results may have important clinical implications in that they suggest the use of DCE-MRI to identify patients with treatment-resistant tumors that may benefit from additional or alternative therapy targeting hypoxia.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Halle, E. Andersen, M. Lando, G. Hasvold, M. Holdén, R.G. Syljuasen, R. Holm, E. Malinen, H. Lyng
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Andersen, G.B. Kristensen, H. Lyng
Study supervision: H. Lyng

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**References**

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27. Law AY, Wong CK. Stanniocalcin-2 is a HIF-1 target gene that promotes cell proliferation in hypoxia. Exp Cell Res 2010;316:466–74.


Hypoxia-Induced Gene Expression in Chemoradioresistant Cervical Cancer Revealed by Dynamic Contrast-Enhanced MRI

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