Hypoxia-induced gene expression in chemoradioresistant cervical cancer revealed by dynamic contrast enhanced MRI

Cathinka Halle¹, Erlend Andersen², Malin Lando¹, Eva-Katrine Aarnes¹, Grete Hasvold¹, Marit Holden³, Randi G. Syljuåsen¹, Kolbein Sundfør⁴, Gunnar B. Kristensen⁴,⁵, Ruth Holm⁶, Eirik Malinen², Heidi Lyng¹.

Authors’ Affiliations: ¹Department of Radiation Biology, ²Department of Medical Physics, ⁴Department of Gynaecologic Oncology, ⁵Institute for Medical Informatics, ⁶Department of Pathology, The Norwegian Radium Hospital; ³Norwegian Computing Center, Oslo, Norway

Corresponding Author: Heidi Lyng, Department of Radiation Biology, The Norwegian Radium Hospital, Nydalen, 0424 Oslo, Norway; Phone: 47 2278 1478.

E-mail: heidi.lyng@rr-research.no

Running title: Molecular background of DCE-MRI in cervical cancer

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.
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. Based on 78 cervical cancer patients subjected to curative chemoradiotherapy, we identified the prognostic DCE-MRI parameter $A_{\text{Brix}}$ by pharmacokinetic analysis of pretreatment images based on the Brix model, where tumors with low $A_{\text{Brix}}$ appeared to be most aggressive. Gene set analysis of 46 tumors with pairwise DCE-MRI and gene expression data showed a significant correlation between $A_{\text{Brix}}$ 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 (UPR), were the most significant. In the remaining 32 tumors, low $A_{\text{Brix}}$ was associated with upregulation of HIF1α protein expression, as assessed by immunohistochemistry, consistent with increased hypoxia. Based on the hypoxia gene sets, a signature of 31 genes that were upregulated in tumors with low $A_{\text{Brix}}$ 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 non-invasively identify patients with hypoxia related chemoradioresistance.
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 biological information related to tumor physiology (2). It is a growing interest to utilize 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 due to 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 utilized, partly because the biological 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). Biological 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 since the concentration of contrast agent in blood; i.e., 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 the present work, 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 performed pharmacokinetic analyses of pretreatment DCE-MR images and generated parameter
histograms of each tumor to account for the intratumor heterogeneity in physiological conditions. For the most prognostic parameter, the percentiles showing the strongest association with survival were used to extract a DCE-MRI parameter which 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 demonstrates 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 187 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 pathological lymph nodes in the pelvis at the time of diagnosis were evaluated by MRI, according to the response evaluation criteria in solid tumors (RECIST) 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 pelvis and retroperitoneum as well as X-ray of thorax were performed. Relapse (progressive disease) was classified as locoregional (regression within the irradiated field), distant, or both.

One to four tumor biopsies, approximately 5x5x5 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, Milwaukee, Wisconsin) with a pelvic phased array coil was used for MRI. Standard T1- and T2-weighted imaging was performed in addition to DCE-MRI. To record the DCE-MRI series, an axial T1-weighted fast spoiled gradient recalled (FSPGR) sequence was utilized (7). Gd-DTPA (0.1 mmol/kg body weight; Magnevist®; Schering, Berlin, Germany) 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 one series was recorded before the bolus injection and 13 after. The temporal resolution was 15 s for the first 11 image series and 1 min for the remaining two 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) (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:

\[
RSI(t) = \frac{S(t) - S(0)}{S(0)}
\]

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:

\[
RSI(t) = A_{Brix} \cdot \frac{k_{ep}}{k_{el} - k_{ep}} \cdot (e^{-k_{ep}t} - e^{-k_{el}t})
\]

where \(A_{Brix}\) 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 \(A_{Brix}, k_{ep}, k_{el} \geq 0\). 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). Based on their n th percentile value, the patients were separated into two equally sized groups, and log-rank tests were employed to assess whether the risk of relapse differed significantly between the two 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; i.e., with the strongest association to outcome (Fig. 1C). For $k_{\text{ep}}$ and $k_{\text{el}}$, the associations with survival were non-significant 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 CaSki 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 prior to the experiments by STR profiling using Powerplex 16 (Promega, Madison, WI), 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 (FCS) and 100 U/ml penicillin/streptomycin (Gibco) under a 5% CO2 atmosphere at 37°C. Cells were plated out in plastic dishes (3x10^5 cells for 6 cm dishes, 9x10^5 for 10 cm dishes) 24 h prior to exposure to hypoxic (0.2% O_2, 5% CO_2) or normoxic (95% air, 5% CO_2) conditions for 24 h at 37°C. The hypoxia treatment was performed in an Invivo200 chamber (Ruskinn Technology Ltd, Bridgend, UK) with accurate O_2 and CO_2 controls. The treatment conditions of 24 h with 0.2% O_2 were selected to mimic conditions with prolonged hypoxia and ensure a response by HIF1α and possibly by the unfolded protein response (UPR) (15).
The cell cycle distribution was analyzed on an LSR II flow cytometer (Becton Dickinson, Belgium) after fixation in 70% ethanol and staining with 1.5 μg/ml Hoechst 33258. Western blots were performed by lysing cells with 10 mM Tris HCl lysis buffer (pH 7.5) containing 2% SDS and 100 μM Na3VO4. The proteins were separated by 8% Tris-HEPES-SDS polyacrylamide gels (Pierce Biotechnology, Rockford, IL), blotted on a PVDF membrane and stained with the monoclonal mouse HIF1α antibody clone 54 (1:800, no. 610958, BD Transduction LaboratoriesTM, NJ) and donkey anti-mouse secondary antibody (715-001-003; Jackson ImmunoResearch Laboratories, Inc. West Grove, PA).

**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 performed, using the Illumina bead arrays human WG-6 v3 (Illumina Inc., San Diego, CA) with 48803 transcripts (16). In brief, total RNA was isolated from the frozen tumor specimens using Trizol reagent (Invitrogen, Carlsbad, CA) and from the cell lines using RNeasy MiniKit (Quiagen). cRNA was synthesized, labeled, and hybridized to the arrays. Signal extraction and quantile normalization were performed 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 biological 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 to 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) (17). Further, 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 utilized for a supervised analysis on sixteen gene sets comprising biological processes that were significant in the GO analysis. Seven of the gene sets have been published previously (19-24), five sets were based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathway databases and four sets were generated from the gene expression data of the hypoxia treated cervical cancer cell lines (Supplementary Methods S1). Since multiple gene symbols may exist for the same genes, the symbols in the various gene sets were altered to match those in our Illumina data set and only genes that could be found in our data set were included in the final gene sets (Supplementary Methods S2). For the gene set analysis, the patients were classified into a low $A_{\text{Brix}}$ group or a high $A_{\text{Brix}}$ group, based on the median value of $A_{\text{Brix}}$ in the 78 DCE-MRI patients. The Illumina data file was reduced to one probe per gene by selecting the probe with the greatest average difference between the low and high $A_{\text{Brix}}$ groups.

**Immunohistochemistry**

Immunohistochemistry (IHC) was performed on 32 selected tumors with the monoclonal mouse HIF1α antibody clone 54 (1:25, no. 610958, BD Transduction Laboratories TM, NJ), binding the amino acid 610-727 on the HIF1α protein. The tissue sections (4μm) were stained using the Dako EnVisionTM Flex+ System (K8012, Dako, Glostrup, Denmark). For antigen retrieval, the PT-Link (Dako) and EnVisionTM Flex target retrieval solution at a high pH were utilized, and the sections were incubated with the HIF1α antibody for 30 minutes. A cervical tumor known to express HIF1α was utilized as a positive control, while 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 based on the percentage of positive tumor cells: 0, 0%; 1, 1-10%; 2, 11-25%; 3, 26-50%; 4, 51-75%; 5, >75%. The cytoplasmic staining was generally weak or absent, and was therefore not quantified. The scoring was performed by an experienced scientist at the Department of Pathology (R.H) who was blinded to the DCE-MRI data.

**Statistics**

Mann-Whitney $U$ test was used for comparison of the protein expression level of HIF1α in the patient group with low 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 6-59th 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 8-36th percentile had a significance level below 0.05, whereas the 20-30th percentiles were the most significant ones (RR<0.44, \( P <0.018 \)) (Fig. 1C). The mean of \( A_{\text{Brix}} \) values over the 20-30th 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 to the others, and the probability of survival differed with more than 30% at 5 years follow-up between the two groups (\( P =0.004 \); Fig. 1D).

**Low \( A_{\text{Brix}} \) associates with upregulation of hypoxia response genes and HIF1\( \alpha \) protein**

To examine whether the differences in \( A_{\text{Brix}} \) among the tumors were reflected in the transcriptional program of specific biological 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). Based on these results, a
supervised gene set analysis was performed on all genes on the array to more specifically investigate phenotypes that could be reflected in $A_{Brix}$. The correlation between $A_{Brix}$ 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 non-homologous 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, since the protein modification process was one of its significant sub-categories. 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 $P=0.032$) (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 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. HIF1$\alpha$ protein was upregulated in all three cell lines by the hypoxia treatment, whereas only minor changes were observed in the cell cycle distributions (Supplementary Fig. S2A, B). Four different gene sets were generated from the hypoxia induced expression changes; genes upregulated in all three 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, S2).
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 $A_{\text{Br}}x$ and hypoxia, and showed that the cervical cancer specific gene sets could be useful in the construction of an $A_{\text{Br}}x$ gene signature.

In order to confirm the above finding of a correlation between $A_{\text{Br}}x$ and hypoxia, protein expression of HIF1$\alpha$ was assessed by immunohistochemistry in the 32 remaining DCE-MRI patients who had not been evaluated in the gene expression study. HIF1$\alpha$ was selected as hypoxia marker, since a hypoxia induced HIF1$\alpha$ response was observed in the cell lines (Supplementary Fig. S2B). The patients with low $A_{\text{Br}}x$ had a significantly higher HIF1$\alpha$ expression compared to those with high $A_{\text{Br}}x$ ($P=0.004$) (Fig. 2A-C). In accordance with this result, several of the HIF1$\alpha$ targets were upregulated in tumors with low $A_{\text{Br}}x$ in the DCE-MRI patients used for gene expression analysis. We thus concluded that low $A_{\text{Br}}x$ was associated with tumor hypoxia and upregulation of hypoxia response genes.

$A_{\text{Br}}x$ 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 $A_{\text{Br}}x$, we selected the four significant hypoxia gene sets (Table 1), and extracted the 31 genes with a negative correlation between the gene expression and $A_{\text{Br}}x$; i.e., the genes that were upregulated in tumors with low $A_{\text{Br}}x$. Most of these 31 genes are known to participate in biological processes affected by hypoxia, such as energy metabolism, cell cycle, and proliferation, including HIF1$\alpha$ targets like $ALDOA$, $STC2$, and $HMOX1$ (27-29), and genes involved in the UPR ($DDIT3$, $STC2$, $ERO1L$, and $AK2$) (30-32) (Table 2), suggesting that several parts of the hypoxia induced transcriptional program were reflected by $A_{\text{Br}}x$.

To ensure that the signature sufficiently represented the $A_{\text{Br}}x$ parameter, we performed unsupervised clustering of the 46 DCE-MRI patients based on the expressions of the 31 genes. Clustering showed two groups with different outcome, for which the one with high expression of hypoxia responsive genes, had low $A_{\text{Br}}x$ ($P<0.001$) and poor outcome ($P=0.011$) as compared to 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 $A_{\text{Brix}} (P<0.001)$ and poor outcome ($P=0.011$) compared to the others (Fig. 3B). The DCE-MRI hypoxia gene signature therefore seemed to satisfactorily represent the $A_{\text{Brix}}$ parameter, including its relationship to clinical outcome.

The prognostic impact of the gene signature was further validated in an independent cohort of 109 cervical cancer patients. In this validation set, the patients who clustered together due to 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 to 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 performed 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 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-MR 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 $A_{\text{Brix}}$ and tumor hypoxia were provided by our work. The hypoxia gene sets were significantly associated with $A_{\text{Brix}}$, and revealed upregulation of hypoxia response genes including HIF1α targets in tumors with low $A_{\text{Brix}}$. In line with this, tumors with low $A_{\text{Brix}}$ had higher protein expression of HIF1α compared to those with high $A_{\text{Brix}}$. The results from the GO analysis were also in accordance with these conclusions, as the
biological processes metabolism, cell cycle and DNA damage signaling, which were associated with $A_{\text{Brix}}$, are important targets for the hypoxia response (25, 28, 34). In particular, genes from the metabolism category encoding the glycolytic enzymes ALDOA, GAPDH and PFK4B4 were found to be upregulated in tumors with low $A_{\text{Brix}}$, consistent with the increased glycolytic activity often seen in hypoxic tumors (28). Loncaster et al. (5) showed a relationship between $A_{\text{Brix}}$ and oxygen tension in cervical tumors, as measured by Eppendorf pO$_2$ histography, supporting our conclusion.

Extraction of genes from the hypoxia gene sets that were upregulated in tumors with low $A_{\text{Brix}}$, 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 $A_{\text{Brix}}$ provides information of disease progression that is not covered by the conventional clinical parameters. Additionally, the gene signature appeared as the most significant factor for locoregional control, indicating that hypoxia as measured by $A_{\text{Brix}}$ could be a well needed biomarker of local recurrence in cervical cancer. Furthermore, while previous studies have shown 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 $A_{\text{Brix}}$. The response to hypoxia occurs through different pathways, and may involve activation of HIFs, the UPR or ER stress response, and signaling through the mammalian target of rapamycin (mTOR) kinase (32). Eight of the 31 genes in the DCE-MRI hypoxia gene signature are known HIF1$\alpha$ targets, namely ALDOA, ERO1L, GAPDH, PFKFB4, P4HA2, C4orf3, HMOX1, and STC2 (27, 29, 32, 35, 36), while SCARB1 is a target of HIF2 (the endothelial PAS domain protein 1, EPAS1) (36). The protein encoded by one of the other genes, RHOC, is indirectly associated with HIF1$\alpha$ through its interaction with the von Hippel-Lindau tumor suppressor (VHL) (37), which degrades HIF1$\alpha$. Additionally, AK2, ERO1L, DDIT3 (also known as CHOP), and STC2 have been found to be involved in the UPR (30-32, 38). It thus appears that processes associated with both HIF activation and UPR may be important in the
A hypoxic phenotype depicted by low $A_{\text{Brix}}$. The involvement of HIF activation was further supported by the high HIF1$\alpha$ 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), while 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 HIF1$\alpha$ or ER stress during hypoxia (27, 31, 50). DDIT3 on the other hand, is a pro-apoptotic 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 two 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 since MRI is already in routine use for patient diagnosis at many hospitals (1). Furthermore, the use of non-invasive imaging to assess the molecular hypoxia phenotype could potentially reduce the need of invasive biopsy procedures and be performed during the course of treatment for response evaluation. $A_{\text{Brix}}$ seems to be a valuable DCE-MRI parameter for this purpose due to 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 demonstrated how non-invasive imaging by means of DCE-MRI and $A_{\text{Brix}}$ assessment may visualize hypoxia and its molecular
basis in chemoradioresistant tumors. Our results may have important clinical implications in that they suggest the use of DCE-MRI to identify patients with treatment resistant tumors which may benefit from additional or alternative therapy targeting hypoxia.

**Grant Support**

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**Reference List**


(27) Law AY, Wong CK. Stanniocalcin-2 is a HIF-1 target gene that promotes cell proliferation in hypoxia. Exp Cell Res 2010;316:466-76.


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<td>CHIANG_LIVER_CANCER_SUBCLASS_PROLIFERATION_UP(20)&lt;sup&gt;d&lt;/sup&gt;</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 x3</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>ISHIGAMI_RADIATION_RESISTANCE</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)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17</td>
<td>0.585</td>
<td>0.585</td>
</tr>
</tbody>
</table>

<sup>a</sup>Gene expression profiles of tumors with high (above median) and low (below median) $A_Brix$ were compared.

<sup>b</sup>Gene sets in bold were created from cervical cancer cell lines in the present work, while 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.

<sup>c</sup>The P-values (P) were adjusted for multiple testing.

<sup>d</sup>Obtained from the Molecular Signatures Database (MSigDB) v3.0.
Table 2: The DCE-MRI hypoxia gene signature

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene symbol</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-acetylglucosaminyltransferase4</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>C20ORF20</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>
</tr>
<tr>
<td>2640048</td>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>0.041</td>
<td>-0.302</td>
</tr>
<tr>
<td>6660601</td>
<td>HMOX1</td>
<td>Heme oxygenase (decycling) 1</td>
<td>0.024</td>
<td>-0.333</td>
</tr>
<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-biphosphatase 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>
</tr>
<tr>
<td>2760427</td>
<td>PYGL</td>
<td>phosphorylase, glycogen, liver</td>
<td>0.011</td>
<td>-0.371</td>
</tr>
<tr>
<td>6280632</td>
<td>RPL36A</td>
<td>ribosomal protein L36a</td>
<td>0.041</td>
<td>-0.302</td>
</tr>
<tr>
<td>2190408</td>
<td>UPK1A</td>
<td>uropakin 1A</td>
<td>0.008</td>
<td>-0.383</td>
</tr>
<tr>
<td>830619</td>
<td>DDIT3</td>
<td>DNA-damage-inducible transcript 3</td>
<td>0.004</td>
<td>-0.414</td>
</tr>
<tr>
<td>2760008</td>
<td>KCTD11</td>
<td>potassium channel tetramerisation domain containing 11</td>
<td>0.022</td>
<td>-0.338</td>
</tr>
<tr>
<td>78052</td>
<td>PVR</td>
<td>poliovirus receptor</td>
<td>0.047</td>
<td>-0.295</td>
</tr>
<tr>
<td>4390619</td>
<td>RHOC</td>
<td>ras homolog gene family, member C</td>
<td>0.043</td>
<td>-0.299</td>
</tr>
<tr>
<td>1170170</td>
<td>STC2</td>
<td>stanniocalcin 2</td>
<td>0.007</td>
<td>-0.390</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>$p^d$</th>
<th>Corr$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2100341</td>
<td>C14ORF2</td>
<td>chromosome 14 open reading frame 2</td>
<td>0.030</td>
<td>-0.321</td>
</tr>
<tr>
<td>3460184</td>
<td>C19ORF53</td>
<td>chromosome 19 open reading frame 53</td>
<td>0.017</td>
<td>-0.352</td>
</tr>
<tr>
<td>1980369</td>
<td>C4ORF3</td>
<td>chromosome 4 open reading frame 3</td>
<td>0.049</td>
<td>-0.292</td>
</tr>
<tr>
<td>5900025</td>
<td>FGF11</td>
<td>fibroblast growth factor 11</td>
<td>0.033</td>
<td>-0.315</td>
</tr>
<tr>
<td>1300603</td>
<td>SH3GL3</td>
<td>SH3-domain GRB2-like 3</td>
<td>0.038</td>
<td>-0.307</td>
</tr>
<tr>
<td>5690431</td>
<td>SNTA1</td>
<td>syntrophin, alpha 1 (dystrophin-associated protein A1, 59kDa, 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>
<td>0.011</td>
<td>-0.372</td>
</tr>
<tr>
<td>2970017</td>
<td>S100A2</td>
<td>S100 calcium binding protein A2</td>
<td>0.009</td>
<td>-0.381</td>
</tr>
<tr>
<td>3420671</td>
<td>TRAPPC1</td>
<td>trafficking protein particle complex 1</td>
<td>0.008</td>
<td>-0.384</td>
</tr>
</tbody>
</table>

$^a$Illumina probe ID; $^b$HUGO gene symbol; $^c$Genes in bold are known HIF1α targets, while underlined genes are known to be involved in the unfolded protein response (UPR); $^d$Correlation coefficient (Corr) and P-value ($P$) in Spearman rank correlation analysis of $A_{Brix}$ versus gene expression.
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><strong>Progression Free Survival</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node status</td>
<td>0.007</td>
<td>2.20</td>
</tr>
<tr>
<td>Tumor volume</td>
<td>0.001</td>
<td>3.33</td>
</tr>
<tr>
<td>FIGO-stage c&lt;0.001</td>
<td>3.40</td>
<td>1.93 – 5.97</td>
</tr>
<tr>
<td>Hypoxia-score d0.001</td>
<td>2.90</td>
<td>1.57 – 5.33</td>
</tr>
<tr>
<td><strong>Locoregional Control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node status</td>
<td>0.120</td>
<td>2.27</td>
</tr>
<tr>
<td>Tumor volume</td>
<td>0.102</td>
<td>2.60</td>
</tr>
<tr>
<td>FIGO-stage c0.080</td>
<td>2.48</td>
<td>0.89 – 6.86</td>
</tr>
<tr>
<td>Hypoxia-score d0.033</td>
<td>3.48</td>
<td>1.11 – 11.0</td>
</tr>
</tbody>
</table>

Abbreviations: FIGO, Federation International de Gynecologie et d’Obstetrique; P, P-value; CI, confidence interval.

The analysis was performed on 155 patients, including both the DCE-MRI cohort and the validation cohort; Tumor size was divided into two groups on the basis of the median volume of 43.8 cm³; FIGO stage was divided into 2 groups: 1b-2b and 3a-4a; The hypoxia score was divided into two groups, based on values <0 and >0.
Figure 1. Identification of a prognostic $A_{\text{Brix}}$ DCE-MRI parameter. A, T2-weighted (left), T1-weighted pre-contrast (middle) and T1-weighted post-contrast (right) MR image of a cervical cancer patient with the tumor delineated. B, Tumor $A_{\text{Brix}}$ map superimposed on a T2-weighted MR image (left) and the corresponding cumulative $A_{\text{Brix}}$ histogram with the 20-30\textsuperscript{th} percentiles indicated in blue (right) of the patient in A. Purple color in the $A_{\text{Brix}}$ map represents $A_{\text{Brix}}$ values of 0, red represents values of $\geq 5.0$. C, Relative risk (RR) (blue line) and 95\% confidence interval (CI) (green lines) from log-rank tests, comparing progression-free survival of two equally sized patient groups for each of 100 percentiles of the tumor $A_{\text{Brix}}$ histograms, plotted against percentile (left). The significant 20-30\textsuperscript{th} and 8-36\textsuperscript{th} percentiles are indicated with a red bar and hatches, respectively. The upper 95\% CI limit for significance is marked (dotted line). D, Kaplan-Meier curves for progression-free survival of patients with low (below median) and high (above median) $A_{\text{Brix}}$. $A_{\text{Brix}}$ was calculated from each tumor histogram as the mean value over the 20-30\textsuperscript{th} percentiles. $P$-value from log-rank test and number of patients are indicated.

Figure 2. Association between $A_{\text{Brix}}$ and HIF1$\alpha$ protein expression. A, Tumor $A_{\text{Brix}}$ maps superimposed on T2-weighted MR images of two different cervical cancer patients. Purple color represents $A_{\text{Brix}}$ values of 0, while red represents values of $\geq 5.0$. B, Representative sections showing HIF1$\alpha$ protein expression of the tumors in A. Inset demonstrates nuclear HIF1$\alpha$ expression. Original magnification: x4, inset image: x10. A, B, The panels present a tumor with low (below median, upper panel) and high (above median, lower panel) $A_{\text{Brix}}$, as calculated from the tumor histogram as the mean value over the 20-30\textsuperscript{th} percentiles. C, Box plot (median, first and third quartile) showing nuclear HIF1$\alpha$ protein expression in tumors with low (n=17) and high (n=15) $A_{\text{Brix}}$, respectively. The whiskers extend to the farthest points that are not outliers. The patients are grouped based on the median $A_{\text{Brix}}$ in the 78 DCE-MRI patients. *, $P<0.01$ (Mann-Whitney U-test).

Figure 3. The DCE-MRI hypoxia gene signature in relation to $A_{\text{Brix}}$ and clinical outcome. A, Hierarchical clustering of the 46 cervical cancer patients with both DCE-MRI and gene expression data, based on expression of the 31 genes in the DCE-MRI hypoxia gene signature (left). Box plot of $A_{\text{Brix}}$ (middle) and Kaplan Meier curves for progression free survival (right) of patients in the low (black) and high (red) expression cluster. B, Box plot of $A_{\text{Brix}}$ (left) and
Kaplan Meier curves for progression-free survival (right) of patients with low (green) and high (blue) DCE-MRI hypoxia score. The box plots show median, first and third quartile, the whiskers extend to the farthest points that are not outliers. *, \( P < 0.01 \) (Mann-Whitney U-test). \( P \) -values from log-rank test and number of patients are indicated in the Kaplan Meier plots.

**Figure 4. Validation of the DCE-MRI hypoxia gene signature in an independent cohort.** A, Hierarchical clustering of the 109 cervical cancer patients in the validation cohort, based on expression of the 31 genes in the DCE-MRI hypoxia gene signature (left). Kaplan Meier curves for progression-free survival of patients in the low (black) and high (red) expression cluster (right). B, Kaplan Meier curves for progression-free survival of patients with low (green) and high (blue) DCE-MRI hypoxia score (right). \( P \)-value from log-rank test and number of patients are indicated.
Figure 1

A

B

C

D

High $A_{\text{Brix}} (n=39)$

Low $A_{\text{Brix}} (n=39)$

P = 0.004
Figure 2
Figure 3

(A) Cluster-low expression (n=23) vs. Cluster-high expression (n=23).

(B) Low hypoxia score (n=25) vs. High hypoxia score (n=21).
Figure 4

A

Cluster – low expression (n=60)

Cluster – high expression (n=49)

B

Low hypoxia score (n=53)

High hypoxia score (n=56)

P = 0.002

P = 0.006
Hypoxia-induced gene expression in chemoradioresistant cervical cancer revealed by dynamic contrast enhanced MRI

Cathinka Halle, Erlend Andersen, Malin Lando, et al.

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