

ORIGINAL ARTICLE

Identifying pH independent hypoxia induced genes in human squamous cell carcinomas *in vitro*

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Abstract

Background. Genes upregulated by low oxygen have been suggested as endogenous markers for tumor hypoxia. Yet, most of the genes investigated have shown inconsistent results, which have led to concerns about their ability to be true hypoxia markers. Previous studies have demonstrated that expression of hypoxia induced genes can be affected by extracellular pH (pH_e). **Methods.** Five different human cell lines (SiHa, FaDu_{DD}, UTSCC5, UTSCC14 and UTSCC15) were exposed to different oxygen concentrations and pH (7.5 or 6.3), and gene expression analyzed with microarray (Affymetrix - Human Genome U133 Plus 2.0 Array). **Results.** An analysis of two of the cell lines using SAM identified 461 probesets that were able to separate the four groups “Normal oxygen, normal pH”, “Low oxygen, normal pH”, “Normal oxygen, low pH” and “Low oxygen, low pH”. From here it was possible to identify a fraction of probesets induced at low oxygen independent of pH in these two cell lines, this fraction included HIG2, NDRG1, PAI1 and RORA. Further verification by qPCR highlighted the necessity of using more cell lines to obtain a robust gene expression profiles. To specifically select pH independent hypoxia regulated genes across more cell lines, data for FaDu_{DD}, UTSCC5, UTSCC14 and UTSCC15 were analyzed to identify genes that were induced by hypoxia in each cell line, where the induction was not affected by low pH, and where the gene was not significantly induced by low pH alone. Each cell line had 65–122 probesets meeting these criteria. For genes to be considered as target genes (hypoxia inducible pH independent), genes had to be present in three of four cell lines. **Conclusion.** The result is a robust hypoxia profile unaffected by pH across cell lines consisting of 27 genes. This study demonstrates a way to identify hypoxia markers by microarray, where other factors in the tumor microenvironment are taken into account.

Solid malignant tumors are characterized by an inadequate vascular system, which can give rise to microregional areas deprived in nutrients and oxygen [1,2]. The oxygen deficient hypoxic cells have been shown to have a significant negative impact on outcome [3–7], especially in carcinomas of the uterine cervix and of the head and neck treated with radiotherapy [4–6]. Several genes have demonstrated to be upregulated in hypoxic cells, including hypoxia inducible factor 1 α (HIF1A), carbonic anhydrase 9 (CA9), glucose transporter 1 (GLUT1), and lactate dehydrogenase A (LDHA) [8–15]. Many studies have tested these genes as endogenous reporters of hypoxia. None of these have, however, proven their ability as markers of hypoxia and to predict prognosis (reviewed in [16]). In particular, studies comparing CA9 and GLUT1 both to each other, but also to more conventional measurements of tumor oxygen-

ation status, such as polarographic measurements with the Eppendorf electrode or the binding of the exogenous immunohistochemical hypoxic marker pimonidazole hydrochloride (PIMO), have been controversial [17–21].

Additional attempts to use microarray technology to discover new genes upregulated by hypoxia include an *in vitro* approach where Chi et al. used five different non-carcinogenic cell lines, exposed to different grades of hypoxia (2% and 0.02%) for different time periods [22]. This data set was employed by Seigneuric et al., to identify both an early and a very different late hypoxia response, and to correlate the early hypoxia signature to survival in a cohort of patients with breast cancer [23]. Denko et al. employed a cDNA array containing 6 800 genes to analyze six different cell lines, including the carcinoma cell lines FaDu, SiHa and C33a, under a not clearly defined “long term

hypoxia” to identify 110 genes that were hypoxia-responsive [24]. Winter et al. suggested in 2007 a hypoxic metagene containing 99 genes. Microarray results from 59 head and neck cancer tissue were clustered, and genes displaying similarity with a set of known hypoxia-regulated genes (e.g. CA9 and GLUT1) were included in the metagene [25].

The tumors microenvironment is not only characterized by different grades of hypoxia, but also acidosis is a well-known phenomenon in many tumors. This is both due to a high rate of glycolysis – the so-called Warburg effect [26] – and to the chaotic microcirculation [27–30]. Studies have shown tumor pH in the range of 6.15–7.4 [2,31]. Previously we have demonstrated that extracellular pH influences hypoxia related gene expression to a large extent [13,32], with low pH_e suppressing the hypoxia induced upregulation of gene expression in a number of genes, especially CA9. This may account for the non-consistent correlation these genes have previously shown to PIMO and Eppendorf measurements.

Recently, Chen et al. [33] published a gene expression study with one epithelial cell line exposed to hypoxia and lactic acidosis, pH 6.7. They demonstrated a difference in gene expression in cells subjected to hypoxia and acidosis and to cells subjected to hypoxia alone.

The current study is an *in vitro* approach, where we have used five carcinoma cell lines (SiHa, FaDu_{DD}, UTSCC5, UTSCC14 and UTSCC15) treated with different microenvironmental factors, and analyzed gene expression with microarray to identify a robust hypoxia profile unaffected by pH across cell lines.

Methods

Cell culture and hypoxia treatment

The human uterine cervix squamous cell carcinoma (SiHa) cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The head and neck squamous cell carcinomas FaDu_{DD}, a subline of FaDu (an undifferentiated hypopharyngeal SCC), UtScC5, UtScC14 and UTSCC15 (established from tumors from the tongue by Dr. Reidar Grenman, University of Turku, Finland [34]), obtained from Dr. Michael Baumann. Cells were cultured in 80 cm² flasks (NUNC) in Eagle’s minimal essential medium with glutaMAX I and Earle’s balanced salt solution (Gibco) containing 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% fetal calf serum, 100 000 U/L penicillin, and 100 mg/L streptomycin (SiHa), or in Dulbeccos modified Eagle medium with glutaMAX I containing 1.5g/L sodium bicarbonate, 2% HEPES, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% fetal

calf serum, 100 000 U/L penicillin, and 100 mg/L streptomycin (FaDu_{DD}, UTSCC5, UTSCC14 and UTSCC15), with 5% CO₂ in a well humidified incubator. For experiments, 2 × 10⁵ cells were seeded into 60 mm glass Petri dishes three days prior to experiments, at which time cells were in the log-phase of growth. Medium was exchanged prior to hypoxia treatment. Hypoxia was achieved by continually gassing the cells in an airtight chamber for 24 hours with either atmospheric air, 5%, 1%, 0.1%, 0.01% or 0% oxygen, supplemented with 5% CO₂ and nitrogen, at 37°C. To simulate acidosis, pH of the medium (without sodium bicarbonate) was buffered with 20 mM tris (Hydroxymethyl) aminomethane (TRIS) base (Sigma), 20 mM 2-(N-Morpholino)ethanesulfonic acid (MES) (Sigma) and 0.52 g/l NaHCO₃ (Sigma) and titrated to 6.3 or 7.4. The pH adjusted media was applied immediately before the induction of hypoxia. Cell mortality under all conditions was below 5%.

RNA extraction and oligonucleotide microarray analysis

Immediately after removal from the airtight chamber, media was removed and 350 µl RTL buffer (Qiagen) containing 10 ml/l β-mercaptoethanol (Merck, Germany) was added to the Petri dishes. Cells were homogenized in a QiaShredder filter (Qiagen) at 10 000 rpm for two minutes. RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufactures instructions. RNA eluted in RNase free water was quantified using a spectrophotometer (SmartSpecTM Plus, Biorad). For analysis of global expression levels the samples was analyzed on the Human Genome U133 Plus 2.0 Array (AROS Applied Biotechnology, Aarhus, Denmark). All array data was normalized using Bioconductor 4.0 with the Affy package and MAS 5.0 [35]. For the gene clustering analysis, data, including probesets with known unique gene ID, was analyzed with Gene Cluster 2.11 (Michael Eisen, rana.lbl.gov/EisenSoftware.htm) using Pearsons correlation and complete linkage, Java Treeview (ver 1.1.3) and SAM (ver 3.02).

For the profiling analysis genes were considered according to the following criteria: 1) the gene has a 2-fold or higher expression at 0.1% oxygen pH 7.5 than at 5% oxygen pH 7.5 and the difference of the two is five times above noise level; 2) The gene has a 2-fold or higher expression at 0.1% oxygen pH 6.3 than at 5% oxygen pH 6.3 and the difference of the two is five times above noise level; 3) The gene has a 2-fold or higher expression at 0.1% oxygen pH 6.3 than at 5% oxygen pH 7.5 and the difference of the two is five times above noise level and 4) The gene expression level at 5% pH 6.3 is not more than 50% higher than the difference between 5% pH 7.5 and

0.1% pH 6.3. Noise level was set to 200. Analysis were done in Microsoft Excel.

Reverse transcription and Real Time Polymerase Chain Reaction (Real Time PCR)

cDNA was generated using the High Capacity cDNA Archive kit (Applied Biosystems; ABI) according to the manufacturer's instructions. Total RNA (2 µg) was reverse transcribed using random hexamer primers. To detect transcripts of interest, TaqMan Gene Expression assay (ABI) was used for HIG2 (Hs00203383), NDRG1 (Hs00608389), PAI-1 (Hs01126606), RORA (Hs00536545), TFRC (4333770F) and GUSB (4333767F). For each reaction, cDNA (corresponding to 20 ng RNA), 1x assay mix and 1xTaqman Universal PCR mastermix (ABI) in a total of 25 µl was mixed. Reactions were performed on an ABI Prism 7000 Sequence Detector (ABI). All reactions were performed in triplicate. Results were normalized to the housekeeping gene Transferrin receptor (*TFRC*). This housekeeping gene was selected on the basis of a TaqMan Human Endogenous control plate (ABI) on which cDNA from the different treatments was analyzed against 12 common housekeeping genes [13]. The results were analyzed using the Comparative C_T method.

Mouse xenografts

Xenograft tumors of the FaDu_{DD} cell line were grown by subcutaneously injecting cells into the back of nude mice (NMRI (nu/nu)) which were whole body irradiated with 4 Gy (240 kV Philips X-ray machine) two days before injection. When the tumors had reached ~200 mm³ in size, the mice were placed in a gas chamber and exposed to a continuous flow of either 10% O₂ + 90% nitrogen (hypoxia group) or normal air (control group) at a flow rate of 1.33 L/min as previously described [36]. There were six tumors in each group. After hypoxic treatment, the mice were injected intraperitoneally (i.p.) with 60 mg/kg PIMO 90 min prior to the tumors were excised. The excised tumors were snap frozen in liquid nitrogen. To extract RNA, the tumor tissue was homogenized in RTL buffer (Qiagen) containing 10 ml/l β-mercaptoethanol (Merck, Germany) through an QIAshredder homogenizer spin-column (Qiagen). RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufactures instructions. RNA eluted in RNase free water was quantified using a spectrophotometer (SmartSpecTM Plus, Biorad).

Identification of signaling pathways

The Ingenuity Pathway Analysis software (IPA) (Ingenuity Systems, Mountain View, USA) was uti-

lized to identify networks of interacting genes and other functional groups. A cut-off ratio of 1.5 was used to define genes. Using the IPA Functional Analysis tool, we were able to associate biological functions to the experimental results.

Results

Clustering of hypoxia treated cell lines

Two squamous cell carcinoma cell lines, SiHa (cervical cancer) and FaDu_{DD} (head and neck cancer), treated with different grades of hypoxia and pH was analyzed by Affymetrix Gene Expression Microarray. Data for both cell lines was analyzed by unsupervised hierarchical clustering (Figure 1). This demonstrated that the genes separated into two main groups, representing the two cell lines. Within each cell line, the samples then clustered into high and low pH. Finally, the clustering showed that in both cell lines and both pH groups, 21% and 5% oxygen cluster close together. The same is evident for 0% and 0.1% oxygen. For 1% oxygen there was no clear trend; in the SiHa cells, a high resemblance with 5% oxygen for both low and normal pH was observed, while in the FaDu_{DD} cells the 1% groups with 0% and 0.1% oxygen with normal pH, but with 21% and 5% oxygen at low pH.

Clustering of pH independent hypoxia regulated genes

Based on the unsupervised clustering of the samples, we defined four groups for further analysis, combining both cell lines, "Normal oxygen, normal pH" (21%, pH 7.5; 5%, pH 7.5), "Low oxygen, normal pH" (0.1%, pH 7.5; 0%, pH 7.5), "Normal oxygen, low pH" (21%, pH 6.3; 5%, pH 6.3), "Low oxygen, low pH" (0.1%,

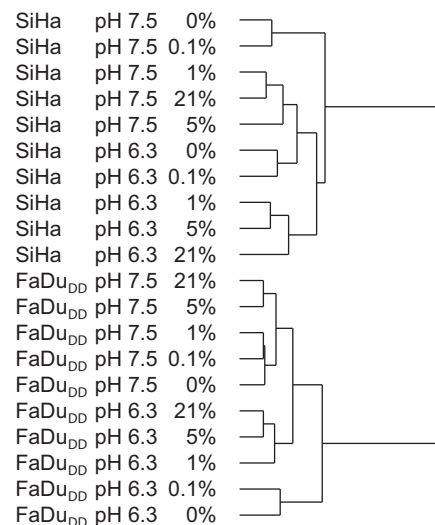


Figure 1. An unsupervised hierarchical clustering of normalized data from SiHa and FaDu_{DD} cells treated with hypoxia and low pH.

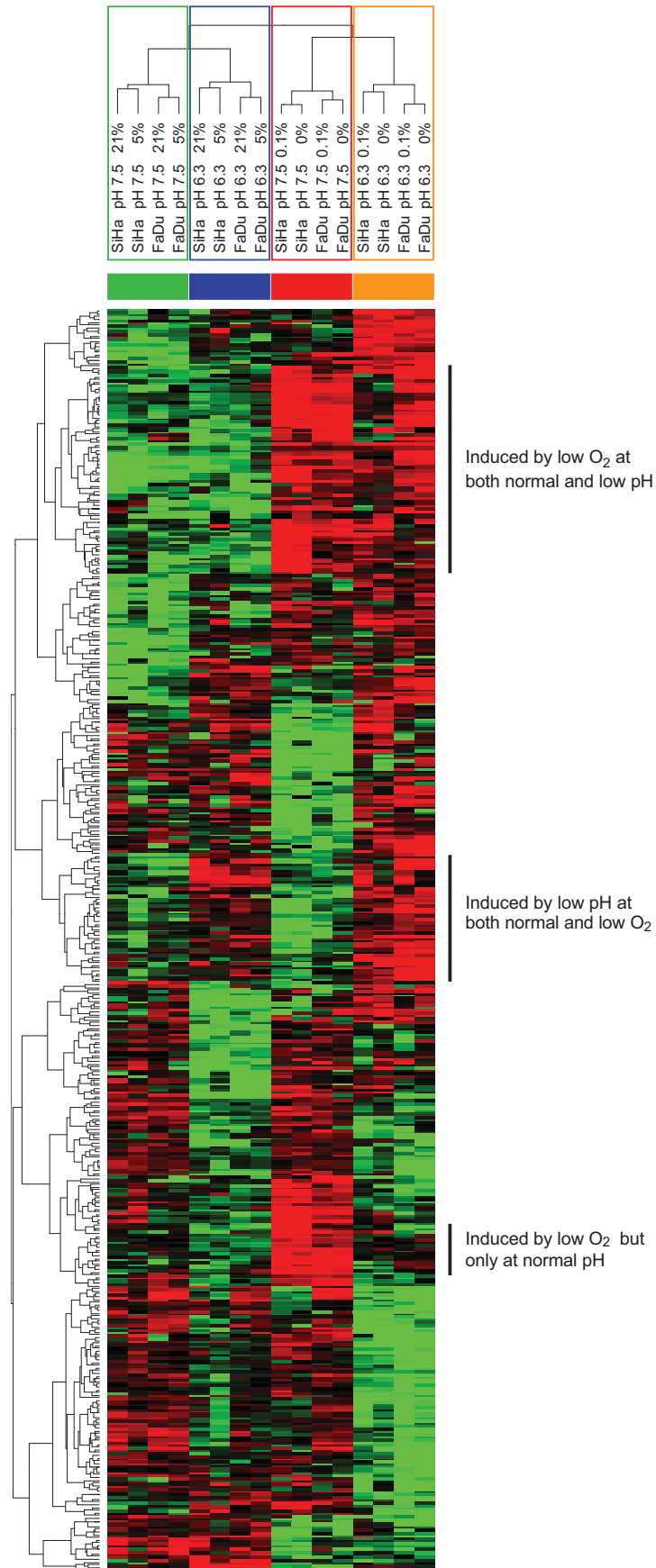


Figure 2. Separating pH independent hypoxia upregulated genes in SiHa and FaDu_{DD} cells. The grouping of samples for the multiclass SAM analysis enabling a hierarchical clustering (median centered genes, complete linkage) of data. Red is upregulated, green is down regulated.

pH 6.3; 0%, pH 6.3) (Figure 2). The samples treated with 1% oxygen did not, based on the previous unsupervised clustering, fit in to these groups, and was therefore not included in the further analysis.

Using a multiclass Significance Analysis of Microarray (SAM) of probesets expressed above the 25% quartile in more than half of the samples and a false discovery rate of 15%, 461 probesets were identified that were able to separate the four groups. A hierarchical clustering (median centered genes, complete linkage) of samples based on the selected probesets is illustrated in Figure 2. Most of the genes identified this

way were genes suppressed by low pH, both at normal and low oxygen. However, it was possible to select a fraction of probesets induced at low pH independent of oxygen concentration (i.e., probesets upregulated in the two sample groups “Normal oxygen, low pH” and “Low oxygen, low pH”). It was also possible to identify a fraction of probesets induced at low oxygen only at normal pH (i.e. probesets upregulated only in the sample group “Low oxygen, normal pH”). Finally, probesets induced at low oxygen independent at pH could be identified. This group consisted of probesets upregulated in both the two sample groups “Low

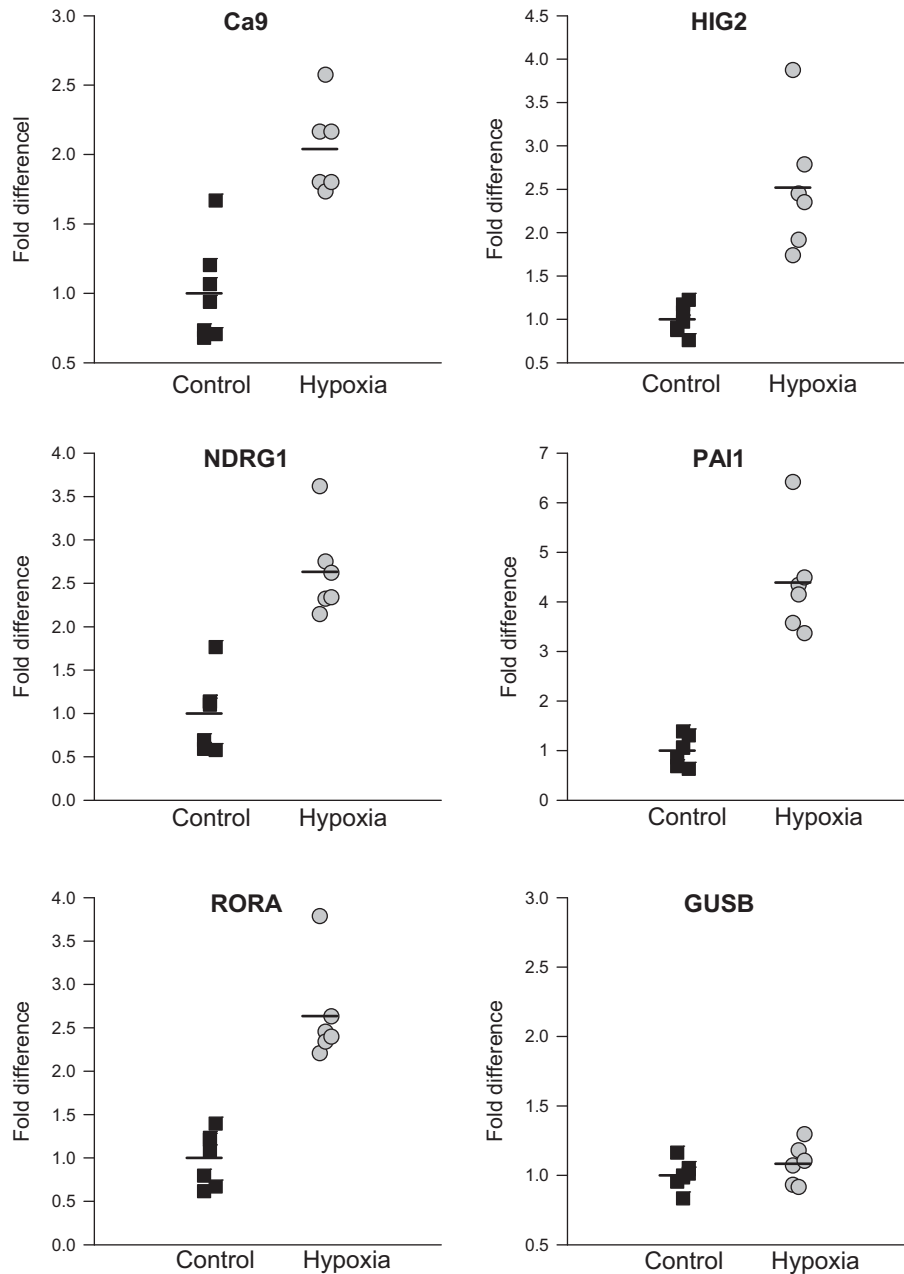


Figure 3. Expression of CA9, HIG2, NDRG1, PAI-1 and RORA in FaDu_{DD} xenograft tumors from mice exposed to either 10% oxygen (hypoxia group) or atmospheric air (control) measured by qPCR. Expression levels are normalized to expression of the housekeeping gene TFRC. Expression of GUSB is measured as control. Each point represents an individual mouse tumor; horizontal bar is the average value.

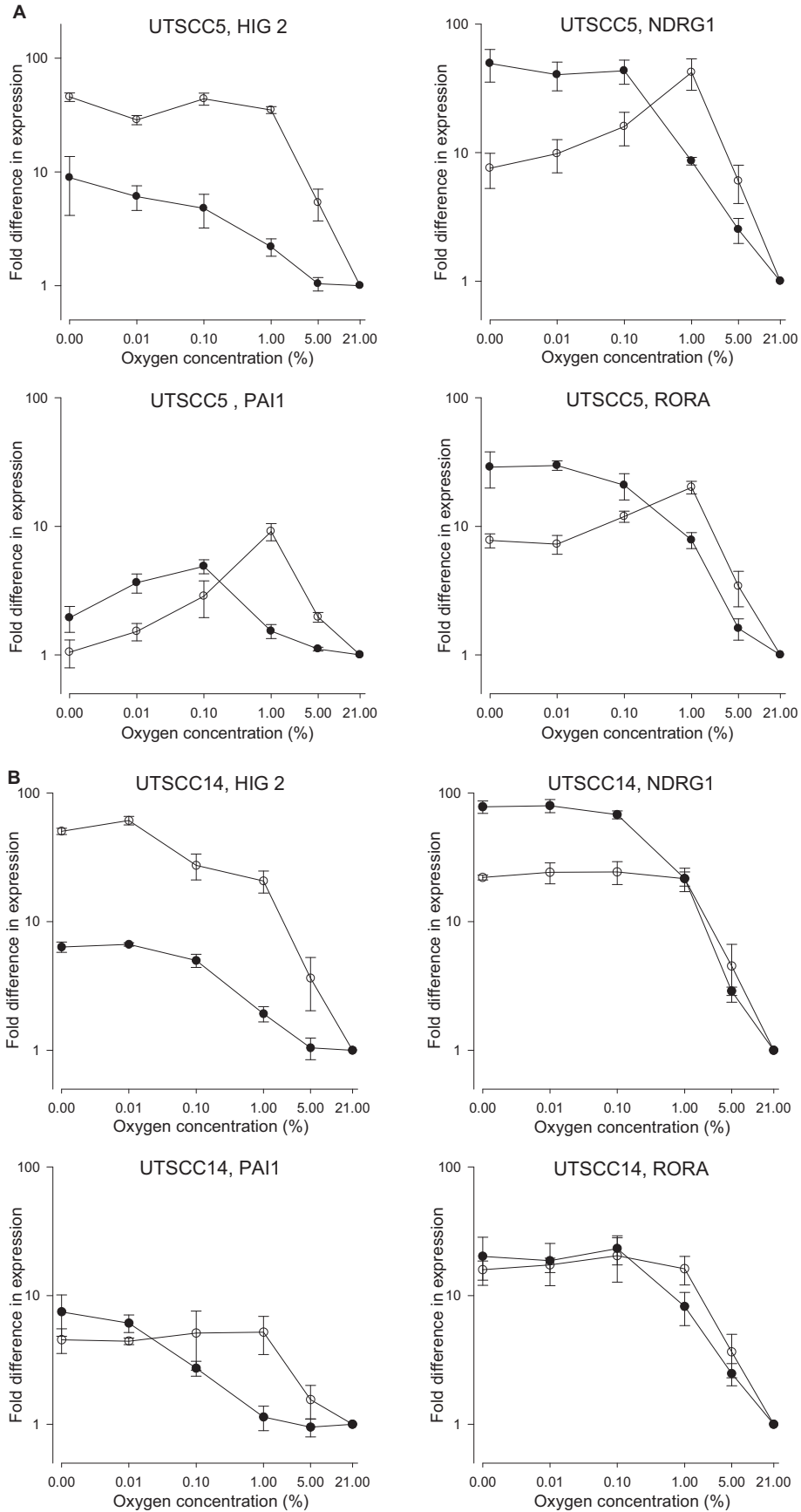


Figure 4. (Continued).

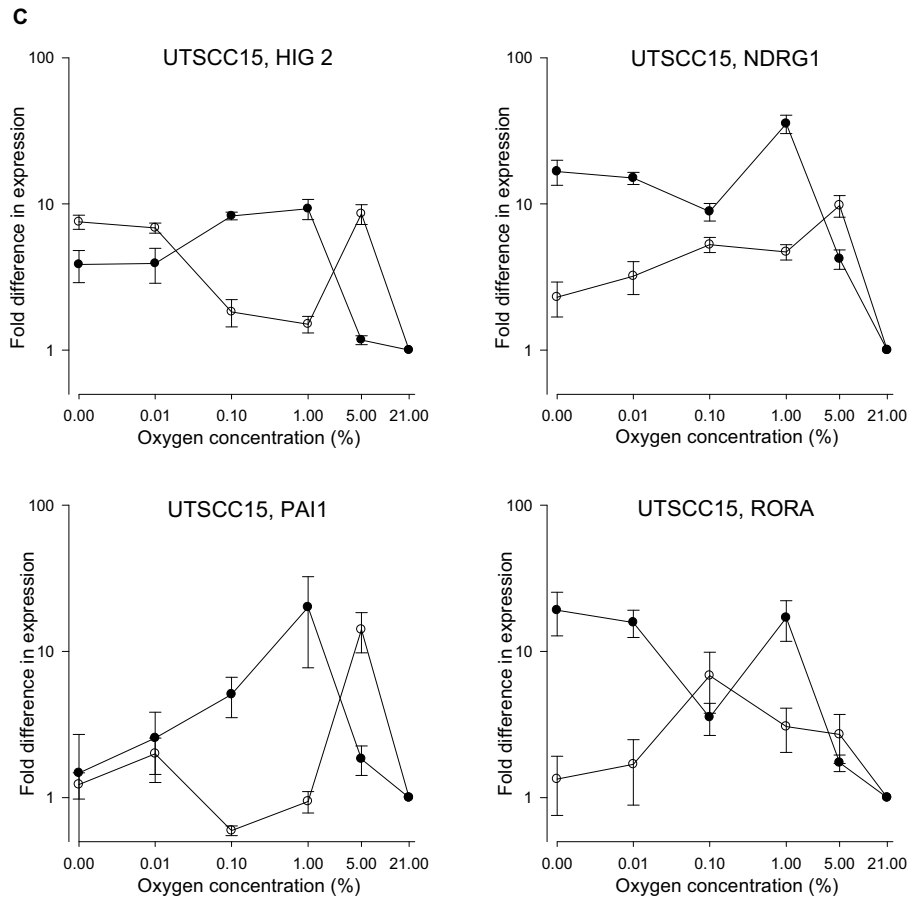


Figure 4. Expression of HIG2, NDRG1, PAI-1 and RORA in A) UTSCC5 B) UtScC14 and C) UTSCC15 cells at different oxygen concentration at either normal pH (●) or low pH (○) measured by qPCR. Expression levels are normalized to expression of the housekeeping gene TFRC, and represented relative to expression levels at 21% O₂. Results show mean (+/- 1 S.E.) from three independent experiments.

oxygen, normal pH” and “Low oxygen, low pH”. CA9 was found amongst the top genes upregulated at low oxygen only at normal pH. Most genes identified also came up when running a similar analysis with array data normalized with RMA (data not shown).

Follow-up of microarray results by qPCR

As a technical control of the microarray analysis, four genes that demonstrated a high SAM score in the fraction of probesets upregulated by hypoxia under both low and normal pH in both MAS and RMA normalized data sets were selected to be validated by qPCR. This was done on RNA from the same experiment as had been used for the microarray analysis (Supplemental Figure 1). These genes were Hypoxia Inducible Gene 2 (HIG2), N-myc downstream regulated gene 1 (NDRG1), SERPINE1 (PAI1) and retinoic acid-receptor-related orphan receptor A (RORA). As a biological control to determine whether the same expression patterns could be found in an *in vivo* system, tumor xenografts of FaDu_{DD} cells were grown on nude mice. The mice were placed in a

pressure chamber to breathe either 10% O₂ (hypoxia group) or normal air (control group) for 24 hours. This has, in other tumor models, been shown to lower the tumor pO₂ significantly [37]. Thereafter, the whole tumor was homogenized and gene-expression was measured by qPCR for HIG2, NDRG1, PAI1, RORA and CA9. GUSB was applied as a control. As can be seen in Figure 3, all five genes exhibit a higher level of gene expression in the hypoxia group compared to the control group. CA9 is the gene least upregulated, with an increase of average ~2-fold in the hypoxia group. HIG2, NDRG1 and RORA show an average increase of ~2.5-fold, while PAI1 shows the highest average increase of ~4.5-fold.

To ascertain whether the expression patterns of these genes were maintained across other cell lines, the experiments were repeated in three independent experiments using UTSCC5, UTSCC14 and UTSCC15 cells (Figure 4). Only HIG2 demonstrated a similar pattern across the cell lines: an upregulation by hypoxia at normal pH in all five cell lines, and an even higher level of hypoxic upregulation at low pH. PAI1, NDRG1 and RORA exhibited cell specific responses.

Hypoxia profile: pH independent hypoxia regulated genes

To specifically select pH independent hypoxia regulated genes across more cell lines, four treatments were applied for further microarray studies of the head and neck cancer cell lines UTSCC5, UTSCC14 and UTSCC 15: 5% oxygen, pH 7.5; 0.1% oxygen, pH 7.5; 5% oxygen, pH 6.3 and 0.1% oxygen, pH 6.3. Data for all five cell lines were analyzed to select genes based on different criteria. For genes to be considered as target genes (hypoxia inducible pH independent), expression had to be at least 2-fold upregulated in 0.1% oxygen at both normal and low pH, and there had to be at least a 2-fold difference between 5% O₂ pH 7.5 and 0.1% O₂ pH 6.3. Furthermore, expression at 5% O₂ pH 6.3 should not exceed 50% of the difference between 5% O₂ pH 7.5 and 0.1% O₂ pH 7.5. When applying these four criteria, 80 probesets came up for SiHa, 65 for FaDu_{DD}, 78 for UTSCC5, 89 for UTSCC14 and 122 for UTSCC15. When looking for common genes across the cell lines using this approach, no genes were common in all five. But when excluding SiHa from the analysis, the remaining cell lines (FaDu_{DD}, UTSCC5, UTSCC14 and UTSCC15) had 13 probesets in common for all four cell lines. For genes to be considered robust target genes, they had to meet the above mentioned criteria in three of four cell lines. This resulted in 37 probesets, representing 27 different genes. These 27 genes are shown in a heat map in Figure 5, in which the fold difference is median centered across all cell lines. It can be seen that the basal expression level of the genes is approximately equal in the four cell lines. Nine of these 27 genes were also found as target genes in SiHa cells.

Pathway analysis

To study the biological context of the genes that was regulated in response to hypoxia and acidosis, the cut-off value was set to 1.5-fold difference, for both up and down-regulated genes (Genes and fold values are shown in supplemental Table). Hereby 44 genes were found upregulated and 92 down-regulated. To study whether these 136 genes interacted biologically they were analyzed via Ingenuity Pathway Analysis. These networks indicated functional relationships between gene products based on known interactions in the literature. Three major regulatory networks with an IPA score ≥ 18 , indicating a less than 10⁻¹⁸ chance than the genes in the network is associated together solely due to random events, were linked to the 44 upregulated genes (Table I), and five networks were linked to the 92 down-regulated genes (Top three networks are shown in Table I). The most affected canonical pathways linked to the upregulated genes were Glycolysis/Gluconeogenesis,

Fructose and Mannose Metabolism, Arginine and Proline Metabolism and HIF1 α Signaling. In total 30 pathways were identified for the upregulated genes.

Discussion

Previous studies have demonstrated that the hypoxia induced upregulation of CA9 and other genes is compromised by low pHe in SiHa and FaDu_{DD} cells [13,32]. Therefore, genes that are upregulated by hypoxia independent of other factors in the microenvironment may be more consistent markers of tumor hypoxia. In this study, total gene expression of five human squamous cell carcinoma cell lines exposed to different oxygen concentrations at either normal or low pHe was analyzed by microarray to identify genes upregulated by hypoxia, independent of pHe.

In data from SiHa and FaDu_{DD} cells, unsupervised clustering revealed a high similarity between

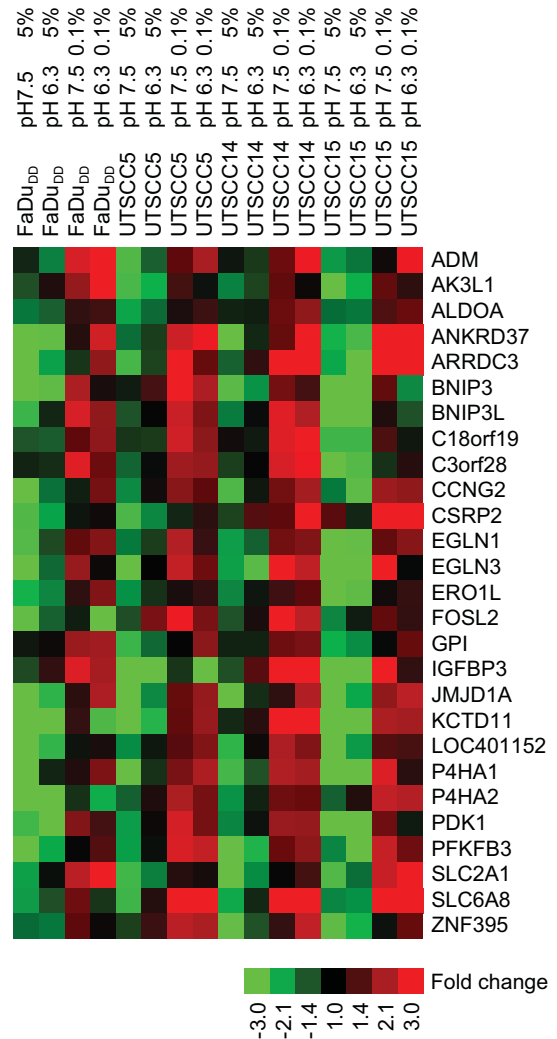


Figure 5. Heat map of the 27 genes that was found as hypoxia induced, pH unaffected in three of four cell lines. The colour bar is showing the fold change relative to the median expression of each gene across all cell lines and conditions.

Table I. The genetic networks affected in hypoxic, acidic cells.

Upregulated genes			
	Genes in Ingenuity networks ^a	Score	Top Functions
1	6-phosphofructo-2-kinase, ADM , Akt, ALDOA , BNIP3 , BNIP3L , CAMK2N1 , CSRP2 , DOK5, ERK, EZR , FOSL2 , FSH, GPI , GPRC5A , hCG, Histone h3, HK2 , IGFBP3 , IgG, Insulin, Mapk, MTUS1 , NFkB (complex), P38 MAPK, P4HA1 , P4HA2 , PDGF BB, PFKFB3 , PGK1 , Procollagen-proline dioxygenase, SAP30 , SLC2A1 , SNED1, TMEM11	47	Carbohydrate Metabolism, Amino Acid Metabolism, Post-Translational Modification
2	AK3L1 , AKAP12, ANPEP, ARRDC3 , ATP1A1, beta-estradiol, citric acid, Co2+, COL1A2, DDX5, DIO2, EGF, EGLN, EGLN1 , EGLN3 , FAM162A , FASN, FKBP8, IFNG, IGFBP4, KCTD11 , Ldh, OS9, PLOD2 , PSMC3, RPS6, SGK3, SIAH2, Smad, SMS , TERT, TNF, VEGFA, WSB1	18	Lipid Metabolism, Small Molecule Biochemistry, Cellular Growth and Proliferation
3	ANPEP, AVP11 , CCNG2 , CDKN1A, COPB2, DDX1, DLST, EEF2, EIF2S3, ERO1L , HNF4A, HNRNPF, HSD17B4, JMJD1A , MAOA, MIR17, MYC, NDRG1 , P4HA1 , PDK1 , PHYHIP, PKM2, PPP2R5B, PSMD2, RBM15, RBPJ , RPN2, RPS9, RPS16, RPS20, S100B, SEC23A, SERTAD2 , SHMT2, TARS	18	Cell Cycle, Hair and Skin Development and Function, Cellular Development
Down-regulated genes			
	Genes in Ingenuity networks ^a	Score	Top Functions
1	C10ORF119 , C16ORF53 , Caspase, CDC6 , CDC45L , CLCF1 , Cyclin A, DMAP1 , DNA-directed DNA polymerase, DTL, E2f, E2F3 , FANCD2 , hCG, Histone h3, ITPKB, MCM3 , MCM7 , MCM10 , NFkB (complex), PARP1 , POLA1, POLA2 , POLE, POLE2 , POLE4, PSME3 , RFC5 , RIF1 , RNA polymerase II, RUVBL1 , SACS , SLC20A1 , STEAP1 , TFPI2	50	DNA Replication, Recombination, and Repair, Cancer, Neurological Disease
2	CCT5 , CRELD1, CSE1L , DKC1, EBNA1BP2 , EEF1G, EIF6, EIF1AX , GTPBP4, HARS , HELLS , IDH3A , IDH3B, IDH3G, IFNB1, IPO13, KIAA1524 , LYAR , MRPL20 , MTAP , MYC, NETO2 , NFYB, NME1, NOP56 , PIH1D1, PPIA, PRPF19 , RPL24, RPL26, RPL32, RPL35, RRP15 , USP13, XPO5	32	RNA Post-Transcriptional Modification, Protein Synthesis, Cell Cycle
3	A1CF, ADAT1 , AKR1C3, CDC45L , DCLRE1B , DTL , FAM98B , FXC1, GAPDH, HNF1A, HNF4A, LAD1 , LARP4 , LEF1, MRPS28 , NAT10, NUFIP1, PIH1D1, PRPF4 , PRPF31 , SART3, SFRS1, SGK1, SLC25A6, SLC25A13 , TCF7L2, THAP10 , TIMM9, TIMM10 , TIMM22, TIMM23, TRMT6 , TTK, WBP4, WDR46	29	Embryonic Development, Tissue Development, Gene Expression

^aGenes in boldface were identified to be expressed differentially more than 1.5-fold.

cells treated with 21% and 5% oxygen, within each cell line. This demonstrates that when performing *in vitro* hypoxia experiments, both of these oxygen concentrations are suitable as controls. This is has been an issue due to normal oxygen levels in tissues being around 5% oxygen, whereas the oxygen concentration for *in vitro* cultures is atmospheric air, 21% oxygen. The 1% oxygen sample falls into either “high” or “low” oxygen, dependent on the cell type. Therefore, as it seems to be cell line specific, the 1% data was not included in the subsequent analyses.

Using a SAM multiclass analysis, it was possible to identify different sets of genes that were associated with the different exposures to oxygen and pH, including a fraction of genes upregulated by hypoxia independent of pH. To test the expression of these genes across more cell lines, four selected genes (PAI1, HIG2, RORA and NDRG1) were analyzed by qPCR. All four genes have previously been reported as hypoxia inducible [22,38–46]. In FaDu_{DD} all four genes were induced by hypoxia both at nor-

mal and low pH, but in UTSCC5, UTSCC14 and UTSCC15 the patterns were more heterogeneous and the genes displayed only partly pH independent hypoxia induction.

An *in vivo* study in FaDu_{DD} xenografts showed an upregulation in hypoxic tumors of these four genes. Furthermore, all four genes were more upregulated than CA9. This is in spite of the findings from *in vitro* studies that have demonstrated CA9 to be up to over 100 times upregulated under hypoxia in FaDu_{DD} cells [32]. This difference could be due to the high heterogeneity in the tumor, with areas not only characterized by low oxygen but also by low pH.

Microarray gene expression data from UTSCC5, UTSCC14 and UTSCC15 were applied, enabling a robust signature across cell lines to be identified. An analysis approach using different criteria was used, making it possible to distinguish genes that in each cell line were induced by hypoxia, where the induction was not affected by low pH, and where the gene was not significantly induced by low pH alone. Each cell line

had 65–122 probesets meeting these criteria, but when looking for common probesets across the cell lines, the probesets of SiHa differed from the other cell lines. Amongst the analyzed cell lines, SiHa is the only cervical cancer, whereas the others all are head and neck cancers. SiHa has also been shown to have a predominantly non-glycolytic phenotype, as opposed to most cancer cells which have a elevated aerobic glycolysis (the Warburg effect) [47]. This might influence this cell lines response to hypoxia. Hence, SiHa was excluded from the analysis. To be considered as hypoxic induced pH independent gene, it had to be common in three of the four cell lines. Thirty seven probesets, representing 27 different genes, met this criterion. These 27 genes compose a hypoxic profile not influenced by microenvironmental factors. The four genes selected from the first analysis, HIG2, PAI1, RORA and NDRG1 were not included in the 27 genes found in the second analysis. All four did, as expected, meet the criterions in the SiHa and the FaDu_{DD} cells, but in the other cell lines HIG2 was upregulated by low pH at normal oxygen concentration. PAI1, in UTSCC 5 and 14, was not induced by low oxygen at either pH, and for RORA the upregulation was compromised at low pH. NDRG1 was included with a cut-off value at 1.5-fold, but not at 2-fold.

Functional interpretation of significant genes in the context of molecular networks and relevance to canonical pathways was generated through the use of Ingenuity Pathways Analysis. The genes consistently upregulated after hypoxic and acidic treatment was detected in pathways related to glucolysis, metabolism and HIF1 α . While most genes were implicated in glucolysis and metabolism pathways, three of the upregulated genes were involved in HIF1 α signaling: SLC2A1 (GLUT1), EGLN1 and EGLN3. The two latter are both prolyl hydroxylases, which contributes to HIF1 α degradation at normoxia, and have previously demonstrated to be upregulated under hypoxia [48].

In conclusion, this *in vitro* study has identified genes that in four human squamous cell carcinoma cell lines are up- or down-regulated under hypoxia without influence of low pH_e. Previously used endogenous marker of hypoxia, such as CA9, can be highly influenced by low pH_e. Due to the Warburg effect and the heterogeneity in tumors, regions with low oxygen levels may overlap with regions of low pH_e. The genes identified here therefore represent novel candidates for endogenous markers of hypoxia, which are not influenced by these local differences in the tumor microenvironment.

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