



Julia Denekamp Award 2015

## The usability of a 15-gene hypoxia classifier as a universal hypoxia profile in various cancer cell types



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### ARTICLE INFO

#### Article history:

Received 28 May 2015

Received in revised form 19 June 2015

Accepted 30 June 2015

Available online 10 July 2015

#### Keywords:

Hypoxia  
Gene expression  
Hypoxia profiles  
Colorectal adenocarcinoma  
Prostate adenocarcinoma  
Esophageal carcinoma

### ABSTRACT

**Background and purpose:** A 15-gene hypoxia profile has previously demonstrated to have both prognostic and predictive impact for hypoxic modification in squamous cell carcinoma of the head and neck. This gene expression profile may also have a prognostic value in other histological cancer types, and could potentially have a function as a universal hypoxia profile. The hypoxia induced upregulation of the included genes, and the validity of the previously used reference genes was established in this study, in a range of different cell lines representing carcinomas of the prostate, colon, and esophagus.

**Materials and methods:** Eleven adenocarcinoma and one squamous cell lines: Six colon carcinomas (HTC8, HT29, LS174T, SW116, SW948 and T48), 3 prostate carcinomas (LNCaP, DU-145 and PC-3) and 3 esophagus carcinoma cell lines (OE19, OE21 and OE33) were cultured under normoxic or hypoxic conditions (0% O<sub>2</sub>) for 24 hours. Total RNA was extracted and gene expression levels measured by qPCR. For each tissue type, individual reference genes were selected and applied in the normalization of the relative expression levels.

**Results:** In all three tissue types, individual, optimal, reference genes were selected. In the analysis of the hypoxia induced genes, both the original reference genes and the new selected reference genes were used. There was no significant difference in the obtained data. The gene expression analysis demonstrated cell line specific differences in the hypoxia response of the 15 genes, with BNIP3 not being upregulated at hypoxic conditions in 3 out of 6 colon cancer cell lines, and ALDOA in OE21 and FAM162A and SLC2A1 in SW116 only showing limited hypoxia induction. Furthermore, in the esophagus cell lines, the normoxic and hypoxic expression levels of LOX and BNIP3 were below the detection limit in OE19 and OE33, respectively. However, a combined analysis of the 15 genes in both adenocarcinoma cell lines and squamous carcinoma cell lines demonstrated a very consistent expression pattern in hypoxic induced gene expression across all cell lines.

**Conclusion:** This study addressed the tissue type dependency of hypoxia induced genes included in a 15-gene hypoxic profile in carcinoma cell lines from prostate, colon, and esophagus cancer, and demonstrated that in vitro, with minor fluctuations, the genes in the hypoxic profile are hypoxia inducible, and the hypoxia profile may be applicable in other sites than HNSCC.

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Hypoxia in solid tumors induces resistance to chemo- and radiotherapy and is a prognostic marker for poor patient outcome in various cancers [1–4]. Several therapeutic modalities have been employed in clinical trials to overcome tumor hypoxia such as hyperbaric oxygen-breathing [5], carbogen and nicotinamide [6], electron affinic radiosensitizers such as nimorazole [7] and hypoxia-selective cytotoxins [8,9]. Addition of hypoxia modifying therapy to conventional radiotherapy was found to significantly improve overall survival in patients with head and neck squamous

cell carcinoma (HNSCC) in a meta-analysis [10]. It remains an important task to identify patients with treatment resistant hypoxic tumors for accurate evaluation of hypoxia modifying strategies. Quantification of endogenous markers expressed by hypoxic tumor cells is a promising strategy for identification of tumor hypoxia. Multiple hypoxia markers have been identified with primary focus on Hypoxia-inducible transcription factor-1 $\alpha$  (HIF-1 $\alpha$ ) [11] and the multitude of HIF-1 activated genes and protein products. Carbonic anhydrase 9 (CA9), osteopontin (OPN), glucose transporter 1 (GLUT1; official gene name SLC2A1) and lactate dehydrogenase (LDH) have all been investigated

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independently, and increased expression has been correlated to tumor hypoxia and poor prognosis [12–16]. However, there has been contradicting results when applying single endogenous markers [17], and in a review of the 15 most promising hypoxia markers, including the before mentioned, no single gene was confirmed as a definitive hypoxia and prognostic marker due to inconsistency in the reported results [18].

It has been suggested that more robust and accurate information about the oxygenation state could be obtained from cumulative quantification of multiple gene expressions [18], and a number of independent hypoxia profiles have been developed, as discussed in [19]. A 15-gene hypoxia classifier was developed by Toustrup et al. for detection of hypoxia in head and neck squamous cell carcinoma (HNSCC) [20]. The optimal genes for the classifier was selected from a panel of 30 validated hypoxia-responsive, pH-independent genes [21]. A training set of 58 HNSCC biopsies, which were previously evaluated as “more” or “less” hypoxic by oxygen electrode measurements [4,22], was used to identify the optimal composition of genes for classification of HNSCCs. The classifier was tested retrospectively and was found to have prognostic impact as well as predictive impact for radiotherapy in conjunction with the hypoxia radiosensitizer nimorazole [23].

There has been an increasing interest in evaluating the hypoxic status in various tissue sites, as for example in prostate cancer [24,25]. A previous study aiming at evaluating the 15-gene hypoxia classifier in patients with loco-regional gastroesophageal cancer did not give a clear answer of the applicability [26]. The purpose of the present study was, in preparation to apply the hypoxia profile in other sites than HNSCC, to investigate whether the genes in the 15-gene hypoxia classifier in a panel of cell lines representing prostate, colon, and esophagus cancer were upregulated by hypoxia in vitro. Furthermore, the usability of the previously used reference genes in the different tissue types was established.

## Materials and methods

### Cell cultures and hypoxia treatment

The colorectal adenocarcinoma cell lines (HCT-8, HT-29, LS174T, SW1116, SW948 and T84) were obtained from Cell Line Service, Germany. The prostate adenocarcinoma cell lines (DU-145, LNCaP and PC-3) were obtained from Dr. Bouchelouche (Department of Clinical Biochemistry, Koege Hospital) and the esophageal squamous cell line (OE21) together with the esophageal adenocarcinoma cell lines (OE19 and OE33) were obtained from Sigma-Aldrich. Cells were cultured in 80 cm<sup>2</sup> flasks (NUNC) in Dulbeccos modified eagle medium (DMEM) with GlutaMAX I containing 4.5 g/L D-Glucose, 10% fetal-calf serum, 1% sodium pyruvate, 1% non-essential amino acids, 2% hepes and 1% penicillin-streptomycin (colorectal and prostate cell lines) or RPMI Medium 1640 with GlutaMAX containing 15% fetal calf serum, 1% penicillin-streptomycin, 1% sodium pyruvate and 1% hepes (esophagus cell lines), with 5% CO<sub>2</sub> in a well humidified incubator. For hypoxia experiments, 200,000 cells (prostate cell lines and esophagus cell lines) were seeded into 60 mm glass petri dishes three days prior to experiments, at which time cells were in the log-phase of growth. For the colorectal cell lines 25,000–50,000 cells were seeded out, depending on growth rate. Hypoxia was achieved by continually gassing the cells in an airtight chamber with 0% oxygen, 5% CO<sub>2</sub> and 95% nitrogen, at 37 °C for 24 hours. This time point was based on previous data for gene expression under hypoxia [27,28]. Normoxic conditions were achieved by gassing cells in an airtight chamber for 24 hours with 95% atmospheric air and 5% CO<sub>2</sub>. Achievement of hypoxic conditions was verified by inclusion of anaerobic indicator strips (Merck, Germany).

### RNA extraction, reverse transcription and gene expression quantification

Immediately after removal from the airtight chamber, media was removed, cells washed with Dulbecco's phosphate-buffered saline (DPBS) and cells lysed with Qiazol Lysis Reagent (Qiagen). Lysis of the colorectal cell lines were performed with RLT buffer containing 10 ml/L β-mercaptoethanol instead of Qiazol Lysis Reagent. Cell lysates were stored at –80 °C.

Total RNA was extracted from cell lysates using the miRNeasy Mini Kit (Qiagen) according to the manufactures instructions. A DNase step was included, according to the manufactures instruction. RNA eluted in RNase-free water was quantified using a NanoDrop 1000 Spectrophotometer at a wavelength of 260 nm (NanoDrop Technologies, Thermo Scientific). Gene expression levels were quantified using Quantitative Real-Time PCR as described in [21]. Briefly, cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ABI) according to the manufactures instructions. Total RNA was reverse transcribed using random primers. Target cDNA transcripts were detected and quantified with TaqMan Gene Expression assays (ABI) (Supplementary information, Tables S1 and S2). For each reaction, TaqMan GeneExpression Master Mix (ABI), cDNA in TE-buffer (Ambion) and the TaqMan Gene Expression assays were mixed. Quantitative Real-Time PCR was performed on a 7900HT Fast Real-Time PCR System (ABI). Genes included in the panel of reference genes were chosen based on previous studies [29–31].

### Data analysis and statistics

The stability of the reference genes was analyzed using the RealTime Statminer (Integromics, Madison, WI), version 20. CT values above 35 were regarded as below detection limit. Thresholds were set manually in the SDS2.1 software. The GeNorm algorithm calculates an internal control gene-stability measure *M*. A low value of *M* means a high relative stability. GeNorm includes the top-ranked genes as reference genes, and the recommended number of reference genes depends on the pairwise variation as described by Vandesompele et al. A pairwise variation under 0.15 was chosen as previously described [32].

The gene expression levels of hypoxia induced genes were calculated using the Comparative CT method [33,34]. ΔCT values were generated by normalizing to the geometric mean of ACTR3, NDFIP1 and RPL37A (reference genes of the hypoxia classifier). Data were also normalized to the three most stable reference genes for each of the examined tissue types. The fold upregulation is calculated as  $2^{-\Delta\Delta CT}$  ( $\Delta\Delta CT = \Delta CT_{\text{Hypoxia}} - \Delta CT_{\text{Normoxia}}$ ).

For the heat map of gene expression levels Gene Cluster 2.11 (Michael Eisen, [rana.lbl.gov/Eisen-Software.htm](http://rana.lbl.gov/Eisen-Software.htm)) (median centered levels) and Java Treeview (ver 1.1.3) was used. Results represent data from three independent experiments.

Pearsons correlations coefficient was used to determine the difference in ΔCt values using different set of reference genes. Box and whiskers plot of gene expression levels were generated in Stata version 14.

## Results

### Optimal reference genes for each tissue type

The expression stability of 22 potential reference genes was analyzed with the GeNorm algorithm. A gene stability measure, *M*, of each included gene was calculated (Supplementary data, Fig. S1). The reference genes analysis was based on normoxic and hypoxic samples, resulting in reference genes where expression

levels were stable under both conditions. GeNorm also calculated the recommended number of reference genes needed for normalization, dependent on the pairwise variation (Supplementary data, Table S3). The optimal sets of reference genes identified were: PSMC4, PUM1, TBP for esophageal carcinoma cell lines; TBP, PSMC4 and NDFIP1 for prostate cancer cell lines; and PUM1, TBP and ACTR3 for colon cancer cell lines.

To evaluate the influence of using the optimally selected set of reference genes for each cancer, compared to using the reference genes originally included in the 15-gene hypoxia profile, the expression data of the 15 genes were normalized to either the GeNorm chosen reference genes or the hypoxia profile reference genes. The obtained  $\Delta$ Ct values were compared using Pearson's correlation (Fig. 1), which demonstrated that for all three cancer types, there was a very strong linear correlation, with  $R^2$  values >0.99, between the two sets of  $\Delta$ Ct values. This validated that each of the sets of reference genes performed equally to the hypoxia profile reference genes, and the data was therefore not influenced

by normalization to the reference gene set from the hypoxia profile, instead of the GeNorm chosen reference genes.

Hypoxia induction of the 15-gene hypoxia profile in vitro

It was possible to measure the expression levels of the majority of the genes included in the 15 gene hypoxia profile in the included cell lines. Exceptions from this was EGLN3 in PC3 (prostate cancer cell line) where gene expression was below the detection level in normoxic samples, and in the esophagus cell lines where LOX expression was below the detection limit in OE19 and BNIP3 in OE33, in both normoxic and hypoxic conditions. Comparison of  $\Delta$ Ct values across cell lines revealed a difference in gene expression levels, in the hypoxia gene profile, in cells grown under normoxic conditions compared to cells grown under hypoxic conditions. In the heat map depicted in Fig. 2, it is shown that almost all of the genes in the classifier contributes to homogeneously colored vertical bars. Exceptions are ALDOA, with low

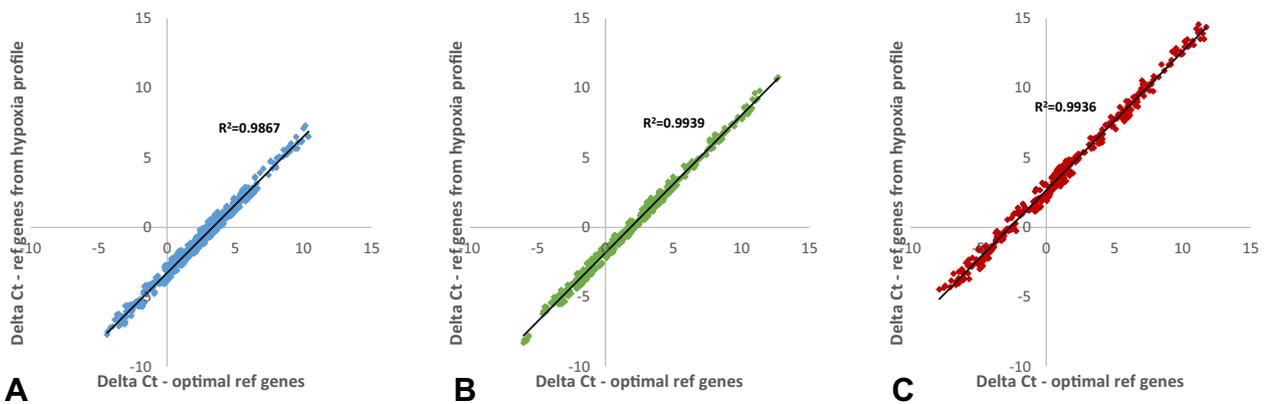


Fig. 1. Comparison of reference genes. The linear correlation of  $\Delta$ Ct values obtained with optimal reference genes vs hypoxia profile reference genes (ACTR3, NDFIP1 and RPL37A) in (A) esophageal carcinoma cell lines (optimal reference genes: PSMC4, PUM1, TBP) (B) prostate cancer cell lines (optimal reference genes: TBP, PSMC4 and NDFIP1) and (C) colon cancer cell lines (optimal reference genes: PUM1, TBP and ACTR3). Pearson's  $R^2$  value is shown.

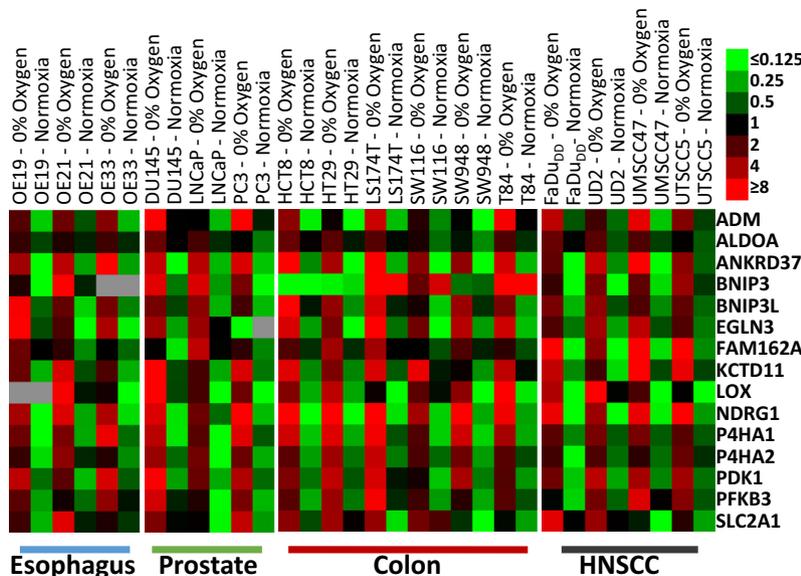


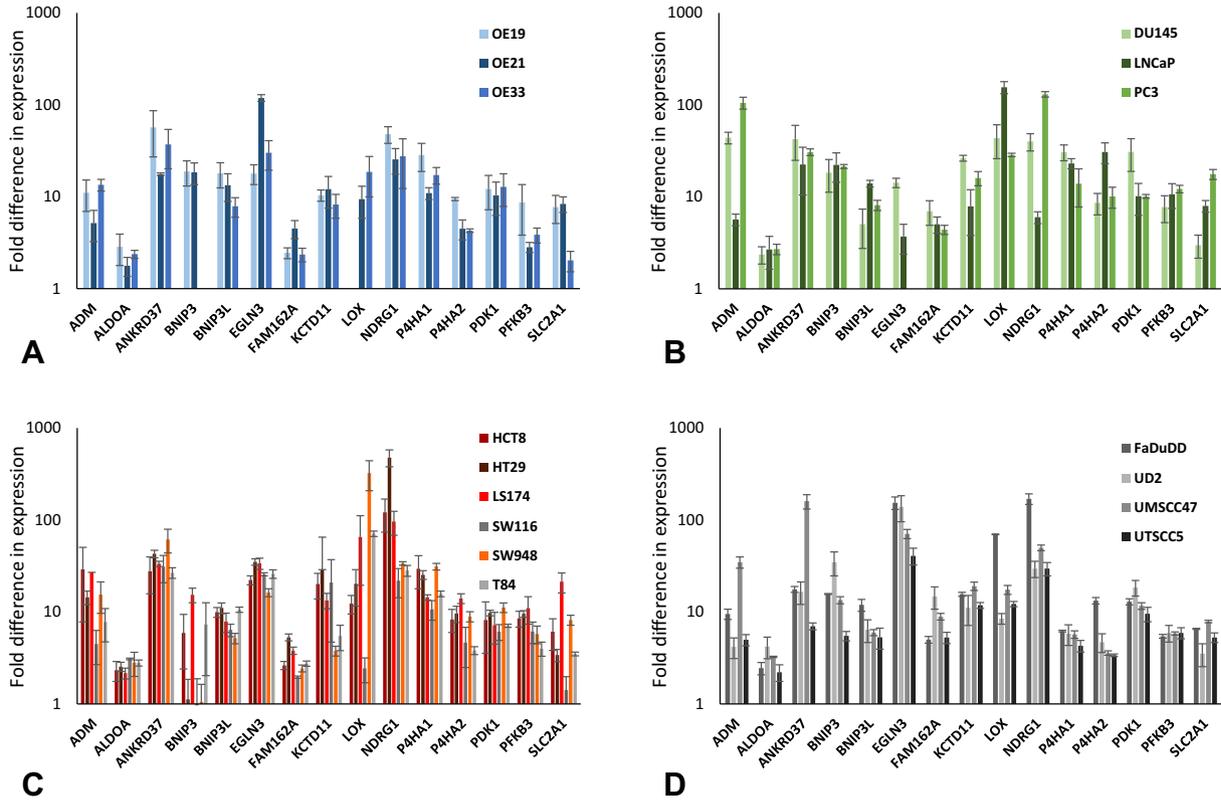
Fig. 2. Gene expression levels in normoxic and hypoxic cells. Heat map of the 15 genes from the hypoxia profile. Genes are median centered within each tissue type. The color bar shows the fold change relative to the median expression of each gene across all cell lines and conditions. Data are mean value of three independent experiments, and are log2 transformed. Expression levels are normalized to expression of the reference genes ACTR3, NDFIP1 and RPL37A. Gray points represents gene expression below the detection limit. For comparison, previous published data on HNSCC cell lines is shown [33].

contrast across cell lines, pointing toward small variations in  $\Delta$ CT in normoxic versus hypoxic samples. BNIP3 presented no effect of hypoxia in colon carcinoma cell lines.

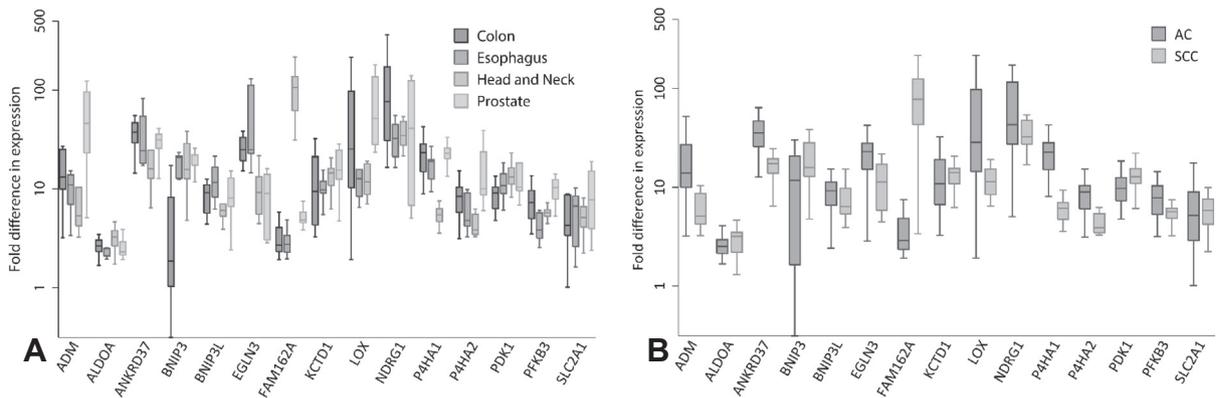
The fold up-regulations in hypoxic samples relative to normoxic samples for each gene are presented in Fig. 3 as the mean value with standard deviations. It is seen from the figure that there is substantial variation in the expression levels of the genes, but the majority are more than two-fold upregulated. Exceptions from

this is BNIP3 which showed very limited upregulation in 3 out of six included colon carcinoma cell lines (HT29, SW116 and SW948), ALDOA in OE21 and FAM162A and SLC2A1 in SW116. ADM, EGLN3, LOX and NDRG1 displays greater than 100 fold upregulation in one or more samples.

The data from the cell lines was combined in an analysis showing the average fold upregulation of each gene across the individual sites and cell types (Fig. 4). Between the four groups,



**Fig. 3.** Relative gene expression under hypoxia for individual cell lines. Fold induction of hypoxia induced genes, relative to the gene expression level under normoxia for (A) esophageal carcinoma cell lines (B) Prostate cancer cell lines and (C) colon cancer cell lines. Expression levels are normalized to expression of the reference genes ACTR3, NDFIP1 and RPL37A. Data are mean values of three independent experiments. Error bars represents standard deviation ( $n = 3$ ). (D) For comparison, previous published data on HNSCC cell lines is shown [33].



**Fig. 4.** Relative gene expression under hypoxia for adenocarcinoma cell lines vs squamous cell carcinoma cell lines. Box plot showing the fold induction of hypoxia induced genes, relative to the gene expression level under normoxia for combined data. (A) Data combined from the individual sites. For comparison, previous published data in HNSCC cell lines is shown [33]. (B) Data combined from adenocarcinoma cell lines (AC) and squamous cell carcinoma cell lines (SCC). For the squamous cell carcinoma cell lines data from OE21 was combined with previous published data from the 4 HNSCC cell lines UD2, UMSCC47, FaDu<sub>DD</sub> and UTSCC5 [35]. Boxes represent the first and the third quartiles, with the median value of the individual independent experiments (the biological replicates for each cell line). Whiskers represents lower and upper adjacent lines ( $n = 14-27$ ).

ANKRD37, KCTD11, NDRG1 and SLC2A1 were not significantly different ( $p > 0.05$ , one-way ANOVA). In order to evaluate combined data from squamous cell carcinoma cell lines, previous published data [35] from 4 HNSCC cell lines (UD2, UMSCC47, FaDu<sub>DD</sub> and UTSCC5) was merged with the data from the esophageal squamous cell carcinoma cell line, OE21. This shows that the 15 genes are mainly consistent in their hypoxia induced expression pattern, with some variation between the cell types in the level of induction. Of the 15 genes, ALDOA, BNIP3L, EGLN3, KCTD11, NDRG1, PDK1 and SLC2A1 were not significantly different between AC and SCC cell lines ( $p > 0.05$ , *t*-test). LOX and BNIP3 in colon cancer cell lines, and, hence, in the combined adenocarcinoma cell lines, display the highest variability.

## Discussion

Reliable determination of gene-expression levels from qPCR requires accurate normalization of target genes to reference genes in order to remove non-biological variation. The expression levels of reference genes often vary between different tissues or under different experimental conditions (reviewed in [36–38]). Therefore, the selection of suitable reference genes is an important key pre-requisite prior to any reliable interpretation of RT-qPCR data, and it is also widely accepted that more than one reference gene may be required [39,40]. We, therefore, wanted to evaluate the use of the three reference genes used with the 15 gene hypoxia profile, as these initially were identified in HNSCC. For this purpose, GeNorm chosen reference genes for each tissue type were identified. The initial panel of 22 reference genes in our study does not make a complete list of all potential reference genes. The influence of normalizing the expression levels of the 15 genes from the hypoxia profile with these individual, GeNorm chosen sets of reference genes, were for each tissue type compared to the data obtained normalizing with the original set of reference genes. The data was not influenced by normalizing it to the reference gene set from the hypoxia profile, instead of to the GeNorm chosen reference genes. This confirms that the reference genes from the hypoxia profile therefore potentially can be used in more tissue types than HNSCC, with the overall constraint that it is based on in vitro data. Optimally, the use of reference genes should be tested in clinical samples as well.

The transcriptional response to hypoxia appears to vary between different human tissues, which may limit universal use of a single hypoxia gene signature [41]. Previous applications of signatures in different tissues did include the Winter metagene, which was derived from HNSCC and did demonstrate prognostic value when applied in breast cancer [42], which opened for the possibility of a universal hypoxia profile. However, a later subset of this metagene, a 26-gene signature developed by Eustace and colleagues, revealed an effect of hypoxia in laryngeal cancer, but not in invasive bladder cancer [43].

One of the included esophageal cell lines in the present study was a squamous cell carcinoma (OE21), whereas the other included cell lines were adenocarcinomas. In our data analysis, there was no difference between the response in OE21 and the adenocarcinoma cell lines, neither in baseline gene expression levels nor in the hypoxia induction of the genes. Data from adenocarcinoma cell lines was merged as well as the data from the squamous cell carcinoma cell line was combined with previous published data for HNSCC. This demonstrated an overall consistent expression pattern in hypoxic induced gene expression across all cell lines.

A universal gene classifier to characterize hypoxic status in various cancers may have important implications in cancer therapy. So far, the use of gene classifiers have been restricted to squamous cell carcinoma of the head and neck, but also in other cancer types

selection of patients with hypoxic cancers for hypoxia modification could be a possibility. In this study, we have addressed the tissue type dependency in hypoxia induction of the genes included in a 15-gene hypoxic profile in cell lines from prostate, colon, and esophagus cancer, and demonstrated that in vitro, with minor fluctuations, the genes in the hypoxic profile are hypoxia inducible. Although this needs to be confirmed in vivo, this work strongly indicates that the hypoxia profile may be applicable in other sites than HNSCC.

## Conflict of interest statement

On the behalf of all co-authors, 'Brita Singers Sørensen' declares no conflict of interest.

## Acknowledgements

The authors would like to thank Lene Bjerre Petersen, Nanna Søndberg Lindberg B Nielsen and Alice Baden for excellent technical help. Financial support was received from the Danish Cancer Society, EC FP7 project METOXIA (project no. 222741), CIRRO – The Lundbeck Foundation Center for Interventional Research in Radiation Oncology and The Danish Council for Strategic Research.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.radonc.2015.06.028>.

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