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Original article

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

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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_2) 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.

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-1α (HIF-1α) [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^2 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_2 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_2 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_2. 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 $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CT_{Hypoxia} - \Delta CT_{Normoxia}$).

For the heat map of gene expression levels Gene Cluster 2.11 (Michael Eisen, 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

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