RESEARCH ARTICLE

Transcriptome sequencing revealed differences in the response of renal cancer cells to hypoxia and CoCl₂ treatment

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Nadezhda Zhigalova*, Artem Artemov*, Alexander M. Mazur, Egor B. Prokhortchouk

Institute of Bioengineering, Research Center of Biotechnology RAS, Moscow, Russian Federation

* Equal contributors

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Abstract

Human cancer cells are subjected to hypoxic conditions in many tumours. Hypoxia causes alterations in the glycolytic pathway activation through stabilization of hypoxia-inducible factor 1. Currently, two approaches are commonly used to model hypoxia: an alternative to generating low-oxygen conditions in an incubator, cells can be treated with CoCl₂. We performed RNA-seq experiments to study transcriptomes of human Caki-1 cells under real hypoxia and after CoCl₂ treatment. Despite causing transcriptional changes of a much higher order of magnitude for the genes in the hypoxia regulation pathway, CoCl₂ treatment fails to induce alterations in the glycolysis / gluconeogenesis pathway. Moreover, CoCl₂ caused aberrant activation of other oxidoreductases in glycine, serine and threonine metabolism pathways.

Keywords

Hypoxia , CoCl₂ , renal cancer , gene expression , metabolism pathways

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1. Alexey Ruzov , University of Nottingham, Nottingham, UK		
2. Colyn Crane-Robinson , University of Portsmouth, Portsmouth, UK		

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Artem Artemov (artem.v.artemov@gmail.com)

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Introduction

Hypoxia is characterized by reduced oxygen supply and appears in multiple pathological conditions including tumours. However, hypoxia can also have a functional role during normal mammalian development and embryogenesis¹. Cells respond to hypoxic conditions both on biochemical and gene expression levels by switching from aerobic metabolism to anaerobic glycolysis and by expression of stress-related genes involved in regulation of cell death, erythropoiesis, angiogenesis and survival²⁻⁴. The activation of many O₂-regulated genes is mediated by hypoxia-inducible factor (Hif1a). Under normoxia, Hif1a is hydroxylated by specific prolyl hydroxylases (PHD1, PHD2 and PHD3). This reaction requires oxygen, 2-oxoglutarate and ascorbate^{5,6}. When Hif1a is hydroxylated, it interacts with the von Hippel-Lindau tumor suppressor protein (pVHL). pVHL forms the substrate-recognition module of an E3 ubiquitin ligase complex, which directs Hif1a poly-ubiquitylation and proteasomal degradation^{7,8}. Under hypoxia (less than 5% O₂), PHD activity is inhibited by cytoplasmic reactive-oxygen species (ROS) which alter the oxidation state of Fe²⁺ (a cofactor for PHD activity) to Fe³⁺. This alteration inhibits PHD activity and Hif1a hydroxylation, thus Hif1a cannot interact with pVHL and promotes Hif1a stabilization^{9,10}. This anaerobic condition and stabilization of Hif1a are characteristic of many tumors. The most common molecular abnormality in renal cell carcinoma is the loss of VHL, which is found in about 50–70% of sporadic cases. Consequently, renal carcinomas with mutations in VHL have high steady-state levels of Hif1a expression and are hypoxic¹¹. Some divalent cations such as cobalt (Co²⁺), nickel (Ni²⁺), and the iron-chelator deferoxamine (DFX), have been applied to mimic hypoxic conditions in cultured cells as they activate hypoxic signals by stabilizing HIF1a¹². Transition metal Co²⁺ could induce hypoxic response by inhibiting PHD activity via iron replacement. Therefore, treatment of a cell culture with cobalt chloride (CoCl₂) is a common model of hypoxia¹³. The second classical setup to study hypoxia is hypoxia induction in a CO₂ incubator with a regulated level of oxygen (less than 1% O₂). In this work, we performed RNA sequencing of Caki-1 clear cell renal cancer cell lines treated with hypoxia and with CoCl₂ to understand how adequate CoCl₂ treatment was as a hypoxia model. We propose that CoCl₂ is not a completely correct model for hypoxia, as it aberrantly induces various hydroxylases not involved in hypoxia pathways and fails to induce downstream biochemical pathways normally induced by hypoxia.

Methods

Cell culture

Caki-1 human clear cell renal carcinoma cells were obtained from American Type Culture Collection (ATCC). Caki-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (GIBCO). For hypoxia treatment, we placed cells into a CO₂ incubator with O₂ control (BINDER CO₂ CB 53) with a regulated environment of 1% O₂, 5% CO₂ and 94% N₂, or cobalt chloride (CoCl₂, Sigma) 300 µM (stock solution 100mM in water) for 24 h.

RNA preparation and RNA sequencing

Total RNA was extracted from Caki-1 cells with Trisol reagent according to the manufacturer's instructions (Invitrogen). Quality was checked with BioAnalyser and RNA 6000 Nano Kit (Agilent). PolyA RNA was purified with Dynabeads® mRNA Purification Kit (Ambion). An Illumina library was made from polyA RNA with NEBNext® mRNA Library Prep Reagent Set (NEB) according to the manual. Sequencing was performed on HiSeq1500 with 50 bp read length. 10 million reads were generated for each sample.

Data analysis

Reads were mapped to hg19 genome (bowtie2-indexed reference downloaded from ftp://ftp.ccb.jhu.edu/pub/data/bowtie2_indexes/hg19.zip) with tophat2 software (version 2.1.0)¹⁴. Gene models of non-overlapping exonic fragments (<http://www.huber.embl.de/pub/DEXSeq/analysis/encode/hsa.DEXSeq.gtf>) were taken from ENSEMBL 54 database (<http://www.ensembl.org/>). For each exonic fragment, total coverage by mapped reads in each sample was calculated with bedtools multicov tool (version 2.17.0). Total gene coverage was calculated as a sum of coverages of all non-overlapping exonic fragments of a gene. Differential expression analysis was performed by applying default read count normalization (estimateSizeFactors) and performing per-gene negative binomial tests (nbinomTest), implemented in DESeq R package (version 1.22.0), with default parameters¹⁵.

We considered a gene to be differentially expressed if the adjusted p-value in DESeq test was lower than 0.05 and fold-change values were higher than 2 (or lower than $\frac{1}{2}$). These sets of differentially expressed genes were further used for gene category enrichment analysis. We took the subset of genes which were found differential in both hypoxia against normoxia controls and after CoCl₂ treatment against normal control and only in one of each experiments. These 3 sets of genes were analyzed with DAVID web service (version 6.7)¹⁶ to find KEGG¹⁷ pathways enriched with the genes.

TCGA data on transcriptomes of kidney tumours (KIRC cohort) was downloaded from Broad Institute FireBrowse (http://gdac.broadinstitute.org/runs/stddata_2015_11_01/data/KIRC/20151101/gdac.broadinstitute.org_KIRC.Merge_rnaseqv2_illumina_hiseq_rnaseqv2_unc_edu_Level_3_RSEM_genes_normalized_data_Level_3.2015110100.0.0.tar.gz). Principal component analysis (PCA) was performed with R prcomp function.

A simple transcriptome-based hypoxia signature was constructed as follows: for every sample being evaluated (e.g., TCGA cancer sample), we considered only the genes which were differentially expressed between hypoxia and untreated Caki-1 cell line (DESeq test adjusted p-value < 0.05). For these genes, we multiplied their logarithmic fold-change (hypoxia vs untreated) to their expression in the evaluated sample. The resulting values were then summed up over the genes under consideration. This yielded a per-sample hypoxia score which would be higher in samples with increased

expression of hypoxia-induced genes and decreased expression of hypoxia-suppressed genes.

Results

Dataset 1. Raw per-gene expression counts for individual genes (see Methods)

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The samples are labelled as untreated_1, untreated_2, untreated_3, CoCl₂_1, CoCl₂_2, CoCl₂_3, hypoxia_1, hypoxia_2, hypoxia_3 which correspond to 3 untreated samples, 3 samples treated with CoCl₂ and 3 samples in hypoxic conditions.

To compare transcriptional effects caused by hypoxia and by CoCl₂ exposure, we performed transcriptome sequencing (RNA-seq) of Caki-1 clear cell renal carcinoma cell line in three conditions: untreated, treated with CoCl₂, and exposed to hypoxic conditions (1% O₂). We searched for differentially expressed genes in two comparisons: CoCl₂-treated against untreated cells and cells under hypoxia against untreated cells.

We first asked how transcriptomic changes after both treatments fit into the established hypoxia gene signature^{1,18,19}. Figure 1A shows relative expression of the genes from hypoxia signature in all sequenced samples. We observed that genes in hypoxia signature were upregulated both in hypoxia and after CoCl₂ treatment, though the effect was much stronger in CoCl₂-treated samples. To

check if a higher magnitude of transcriptomic changes in CoCl₂ compared to hypoxic conditions was characteristic to other differentially expressed genes, we compared the distributions of logarithmic fold-changes (absolute value) for gene expression in these two experiments (Figure 1B). Surprisingly, for the majority of genes, hypoxia-induced changes were higher than the ones induced by CoCl₂. In other words, hypoxia resulted in broader transcriptome response than CoCl₂ treatment even though specific hypoxia-related genes were more affected after CoCl₂ treatment.

These results suggested that CoCl₂ treatment could be an incomplete model of hypoxia capturing only upstream signalling events in hypoxia pathways and not reflecting broader downstream effects. To further investigate the differences between CoCl₂ model and real hypoxia, we compared the sets of differentially up- and down-regulated genes in CoCl₂-treatment (against untreated) and in hypoxia-treated (against untreated) cells. Figure 2A summarizes the overlap between those gene sets. To understand what regulatory and biochemical pathways were affected in each treatment, we performed gene category enrichment analysis over KEGG¹⁷ pathways with DAVID web service (version 6.7)¹⁶ for genes affected in both treatments or exclusively in one treatment.

The genes which were significantly upregulated in hypoxic conditions (1% O₂) but not after treatment with CoCl₂ were significantly enriched in the glycolysis/gluconeogenesis pathway which was known to be related to hypoxia^{1,15,18}. Unexpectedly, we detected no enrichment in glycolysis/gluconeogenesis pathway with the genes

Figure 1. (A) CoCl₂ causes much more pronounced expression changes in the expression of key hypoxia regulators compared to real hypoxia treatment. Heatmap represents relative gene expression for key genes involved in hypoxia regulation. (B) Hypoxia results in broader transcriptome response compared to CoCl₂ treatment, i.e., more genes are changing expression under hypoxia. The figure shows absolute log fold change values for gene expression between hypoxia (or CoCl₂) groups relative to control group. Genes are sorted according to absolute log fold change values. $P(\text{wilcoxon}) < 2.2 \times 10^{-16}$.

differentially expressed after CoCl_2 treatment which was confirmed by a heatmap for glycolysis/gluconeogenesis-related genes (Figure 2B).

The genes upregulated in both hypoxia and under CoCl_2 treatment were enriched in the MAPK pathway. As the MAPK pathway was known to activate hypoxic response, MAPK activation was expected in hypoxia. Even though CoCl_2 affected directly HIF1 α pathway, MAPK was activated in CoCl_2 -treated samples as well as in real hypoxia. This result supported a previous observation of MAPK-dependent activation of hypoxia response under CoCl_2 treatment¹². Surprisingly, we observed systemic lupus erythematosus-related pathway activation in both treatments. The set of genes upregulated in this pathway (histone proteins H2A, H2B, H3, H4 and MHCII antigen-presenting genes) could be unrelated to lupus erythematosus, but rather could indicate increased proliferation and inflammation.

We also explored the genes specific to CoCl_2 treatment but not affected by hypoxia. Surprisingly, the genes upregulated after CoCl_2 treatment but not changed in hypoxia were enriched in the glycine/serine/threonine biosynthesis pathway (Figure 2A and 2B).

We hypothesized that Co^{2+} ion could substitute metal cofactors of several oxidoreductases in the pathway and subsequently impair their activity. This, in turn, could require greater amounts of enzyme to be synthesized.

Hypoxic conditions within a tumour have been shown to predict worse clinical outcome²⁰. We used our data on whole-transcriptome profiling of kidney cancer cell lines in normal and hypoxic conditions to extract a wide hypoxia signature and validate it with TCGA data on transcriptomes of kidney tumours²¹. Major variation in TCGA transcriptomes (Figure 3A) is generated by the difference between tumours and adjacent normal samples. We projected our sequenced samples to the principal components derived from TCGA samples and observed that the direction of transcriptome changes between untreated and hypoxic cell lines is slightly similar to the difference between normal and tumour samples, though hypoxia-related changes couldn't explain normal-tumour difference. We constructed a transcriptome-based hypoxia signature as described in the Methods section. To test if this hypoxia signature predicted clinical outcome for kidney cancer patients, we explored the distribution of our hypoxia scores between disease free patients and patients in which the disease had recurred or

Figure 2. (A) Summary of KEGG pathways, enriched by the genes, up- and down-regulated in CoCl_2 and hypoxia treatment. No enriched pathways were discovered for the genes downregulated in both treatments. (B) Overall expression change in glycolysis/gluconeogenesis (KEGG hsa00260) genes in control, hypoxia conditions and after CoCl_2 treatment. Glycolysis/Gluconeogenesis (KEGG hsa00260) is activated in hypoxia but not after CoCl_2 treatment. (C) Expression change for glycine, serine and threonine metabolism genes (KEGG hsa00260). Glycine, serine and threonine metabolism (KEGG hsa00260) is activated only after CoCl_2 treatment but not under hypoxia.

Figure 3. (A) PCA plot for TCGA samples of kidney tumours, adjacent normal tissue samples, untreated Caki-1 cells, Caki-1 cells treated with CoCl₂ and the cells in hypoxic environment. (B) Hypoxia signature derived from RNA-seq results predicted significantly higher hypoxia scores for recurred or progressed TCGA tumours.

progressed. Hypoxia scores were significantly higher for recurred/progressed patients (Wilcoxon test p-value=0.0009, Figure 3B).

Discussion

The current study for the first time provides RNA-seq data revealing hypoxia-induced transcriptomic changes, which allows broader understanding of the processes related to hypoxia. In our analysis, we explored the limits of applicability of CoCl₂ treatment as the model of hypoxia. Briefly, we observed that CoCl₂ strongly alters expression of few genes important for hypoxia signalling, but fails to influence the essential downstream consequences of hypoxia, particularly the glycolysis/gluconeogenesis pathway. This might suggest the existence of alternative regulation mechanisms which trigger the downstream events in hypoxia together with main VHL/HIF1a pathway. CoCl₂ treatment also aberrantly induced pathways which did not respond to hypoxia. These included the glycine, serine and threonine metabolism pathways. We hypothesized that its aberrant activation might be caused by Co²⁺ ion binding to the enzymes (other than PHD proteins) involved in Glycine, Serine and Threonine biosynthesis.

Data availability

F1000Research: Dataset 1. Raw per-gene expression counts for individual genes (see Methods), [10.5256/f1000research.7571.d109570](https://doi.org/10.5256/f1000research.7571.d109570)²³

RNA-seq data was deposited to NCBI SRA under [SRP066934](https://www.ncbi.nlm.nih.gov/sra/SRP066934) study accession code. The study contained experiments under the following accession codes: untreated (SRX1459966, SRX1459967, SRX1459969), treated with CoCl₂ (SRX1459974, SRX1459977, SRX1459978), exposed to hypoxia (SRX1459979, SRX1459981, SRX1459984).

Author contributions

NZ performed the experiments, AM constructed the libraries for RNA sequencing, AA performed data analysis and prepared the figures, AA and NZ wrote the manuscript, EP designed and supervised the experiment.

Competing interests

No competing interests were disclosed.

Grant information

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I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Colyn Crane-Robinson

School of Biological Sciences, Biophysics Laboratories, University of Portsmouth, Portsmouth, UK

This project uses mRNA sequencing to monitor changes in gene expression in a human kidney cell line in response to direct hypoxia (5% O₂) as compared to treatment with 300mM CoCl₂, frequently used as a surrogate for oxygen depletion. The results are very clear-cut: monitoring several genes known to be key hypoxia regulators shows CoCl₂ to be more effective than oxygen depletion but in contrast, monitoring a much broader gene response showed O₂ depletion to have greater effects. This contrast led the authors to concentrate attention on several specific biochemical pathways: a) glycolysis/gluconeogenesis is strongly activated in hypoxia (as expected) but not following treatment with CoCl₂; b) the reverse is the case for glycine, serine, threonine metabolism which is strongly induced by CoCl₂ but not by hypoxia.

These last examples make clear the non-equivalence of CoCl₂ treatment and real hypoxia and this article therefore issues a strong caveat regarding the use of the inorganic surrogate. It is most certainly worth publishing for this reason alone.

Since hypoxic conditions in tumours are taken to be predictive of a poor clinical outcome, the authors tested whether the direction of changes in the transcriptome of their untreated and hypoxic cell lines mirrors the difference between tumours and their normal cell counterparts. Hypoxia scores from mRNA expression data were somewhat (but significantly) elevated in recurrent/progressive patients. However, this difference does not (unfortunately) appear to be sufficient to use clinically (if I am understanding their intentions correctly). The upshot is that the authors do not mention this comparison in their Discussion.

On practical matters: they might expand the caption to Fig 1. In A) I assume the three tracks are biological replicates: if so, write it. In B) a fuller explanation of the X-axis 'Index' would be helpful. Check on the printing of '300mM CoCl₂' (in Methods, bottom of p2).

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 04 January 2016

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Alexey Ruzov

Division of Cancer and Stem Cells, School of Medicine, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK

In this paper, the authors analysed the response of renal cancer cells Caki-1 to hypoxia and CoCl_2 treatment using whole transcriptome sequencing. The main finding of the manuscript that the profiles of gene expression of Caki-1 cells are qualitatively different under these two conditions, and, therefore, the CoCl_2 treatment may not represent an adequate model mimicking hypoxia, is interesting and merits indexation, in my opinion.

Although, as far as I can ascertain, most of the experiments are technically sound and the main conclusions of the study are justified, I feel that there is a number of issues with the text/interpretation of the data, which need to be addressed to improve the quality of the manuscript:

1. It is not sufficiently clear from the text why the authors have elected the Caki-1 cell line for their study. Is there anything peculiar about these cells? Is VHL mutated/lost/misexpressed in these cells? The last question is especially important since the authors mention that this protein is lost in 50-70% of renal cell carcinomas.
2. According to the Dataset 1, the authors used triplicates for each condition in their analysis. This should be stated in Methods section. The authors should also comment on the nature (biological vs technical) of these replicates there.
3. It is not clear if the numbers presented in the Venn diagram in Figure 2A represent all genes up/down regulated for each condition or only the genes enriched for the indicated KEGG pathways. It is also not clear from the annotation or legend which numbers correspond to upregulated and downregulated genes in this figure.
4. The Discussion is very weak at the moment with no references cited in the section. The authors should rewrite it relating their findings to available literature.

Other points:

1. The abstract lacks a sentence summarizing results of the study and/or pointing at potential implications of the findings for medicine/cancer biology. It would be beneficial for readers if such statement was included in the abstract.
2. The last sentence in Introduction (starting with "We propose...") may well be an

overinterpretation. The conclusion that CoCl_2 treatment is not a "correct model for hypoxia" may need to be additionally confirmed using other cell line systems.

3. Presenting the KEGG enrichment scores for each category alongside the Venn diagram may be helpful for assessing significance of the results.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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