DOI 10.18699/SBB-2022-221

Analysis of hypoxia signature in TCGA-BRCA dataset and in mcf-7 cell line

Brovkina O.*, Yusubalieva G., Nikitin A.
Federal Research and Clinical Center, FMBA of Russia, Moscow, Russia
** brov.olia@gmail.com*

Key words: hypoxia, breast cancer, tumor microenvironment, transcriptome analysis, epithelial-mesenchymal transition, database screening

Motivation and Aim: Hypoxia is a common feature for most solid tumors and is associated with a poor prognosis, while the lack of oxygen enrichment of tumor tissues and their microenvironment reduces the effectiveness of radiation therapy and some chemotherapy drugs [1]. Therefore, the development of clinical drugs that reduce hypoxia, as well as effective and accurate measuring, are being actively pursued [2]. There are several possible methods for detection of hypoxia. One of the most promising methods is gene expression profiling associated with the transcriptional response of a tumor to its hypoxic microenvironment.
Methods: The analysis of large dataset was performed using open database TCGA-BRCA. The analysis included 531 tumor and normal samples. 444 samples were breast carcinoma samples from stage III and IV patients of the disease, 87 samples were normal controls. Differentially expressed (DE) genes were analyzed using the DeSeq2 package. To normalize the obtained data, the TMM (Trimmed Mean of M-values) method was applied with the calculation of CPM (counts per million) taking into account the normalization coefficients. Quasi-likelihood F-test (QLF) and Mann-Whitney U-test (MW) were used to assess the significance of changes in gene expression. High and low expressed genes were selected using p.adj < 0.05 and logFC > 1.5. The fgsea and gprofiler packages were used to enrich the data on DE genes. Among the list of signaling pathways, pathways associated with hypoxia were selected. Then, using the Elastic Net model, the degree of significance of the difference between tumor samples from the norm in terms of DE genes that are part of the hypoxia signaling pathways was determined.
The experimental validation of selected panel of genes was performed on 4T1 cell line. This tumor-derived cell line is an animal model for stage IV human breast cancer. The cells were cultured in RPMI 1640 medium (Life Technologies, USA) supplemented with 5 % fetal bovine serum (FBS) (Life Technologies) and 1 % PenStrep (Life Technologies), in a humidified atmosphere containing 5 % CO_2 and 5 % O_2 in an incubator (Sanyo, Japan) at 37 °C. The cells were subcultured at confluence by treatment with 0.05 % trypsin and 0.02 % EDTA in phosphate buffered saline (PBS). Total RNA isolation from adipose tissue was carried out with a Rneasy Lipid Tissue Mini Kit (Qiagen, Germany) on the automatic QIAcube station according to the manufacturer's protocol. Reverse transcription of RNA was performed using MMLV reverse transcriptase (Evrogen, Russia) and random hexanucleotide primers. Each setting of the reverse transcription reaction included negative controls containing no RNA.
We estimated expression levels of 18 mRNAs by qPCR on a CFX96 thermal cycler (Bio-Rad, USA). A SYBR Green intercalating dye, qPCRmix-HS SYBR (Evrogen), was

added to the PCR mixture. The total volume of the PCR reaction was 20 µl. Conditions for amplification of DNA fragments were: 95 °C for 20 sec – 1 cycle; 95 °C for 1 sec, 60–64 °C for 20 sec – 40 cycles. The B2M gene was a reference gene.

Results: A total of 738 DE genes were identified, of which 499 were up-regulated and 239 were down-regulated. Expression of 36 genes was associated with hypoxia, changes in 50 genes were caused by the process of epithelial-mesenchymal transition (EMT). In addition to previously known genes, the alpha-lactalbumin LALBA gene and the exonuclease gene stimulated by interferon 20 ISG20 were overexpressed. Analysis of signaling pathways revealed the following significant mechanisms: hypoxia-inducible factor-1 (HIF) regulatory pathways, glucose metabolism (SLC2A1, PFKFB4, LDHA, ALDOA, ALDOC), and angiogenesis (ANGPTL4, VEGFA). EMT included the following pathways: fatty acid metabolism (LEP, TGFB, FASN), apoptosis (BNIP3L, BNIP3, NDRG1), Krebs cycle (IDH, SDH), and cell adhesion (SNAIL, TWIST, ZEB1, ZEB2): GO:0001666 GO: 0005975 GO: 0005996 GO: 0006090 GO: 0006091 GO: 0006096 GO: 0033554 GO: 0035694 GO: 0036293 GO: 0036294 GO: 0046031 GO: 0046034 GO: 0046939 GO: 0055086 GO: 0070482 GO: 0071453 GO: 0071456 GO: 0072521 GO: 0072521 GO: 0072521 GO :1901135 GO:0005737

Based on the obtained signatures, we divided the patients into 2 clusters. The first cluster included patients (317/60 %) with high rates of hypoxia (according to the ConsensusClusterPlus program), the second included patients (214/40 %) whose rates were lower. The analysis of survival curves showed that the survival rate of patients with high rates of hypoxia was significantly lower compared to patients from the low hypoxia group. It is noteworthy that in the first cluster patients with metastases predominated, as well as those with impaired EMF mechanisms.

The qPCR analysis confirmed high expression for *LEP, ADM, ALDOA, BNIP3, LOX, NDRG1, P4HA1, PDK1, SLC2A1, LALBA, ISG20* genes (lgFC > 1,5, p.adj < 0,05).

ANRD37, BNIP3L, EGLN3, P4HA2, PFKFB3 genes had no significant difference compared with cell in normoxic condition, while *FAM162A, KCTD11* genes had significantly low expression (lgFC < –1,5, p.adj < 0,05).

Conclusion: The stratification of patients based on the hypoxia signatures makes it possible to identify groups with a high risk of disease progression. Evaluation of gene expression profiles associated with hypoxia allows to detect metastatic potential and select the optimal treatment strategy. The evaluation of individual genes and common pathways in hypoxia is consistent with the global trend towards a personalized approach to the diseases treatment and contributes to the search for targeted therapeutic drugs.

Acknowledgements: The research was funded by the Russian Science Foundation, grant No. 21-75-00041.

References

1. Burroughs S.K., Kaluz S., Wang D., Wang K., Van Meir E.G., Wang B. Hypoxia inducible factor pathway inhibitors as anticancer therapeutics. *Future Med Chem.* 2013;5(5):553-72. doi: 10.4155/fmc.13.17.
2. Bibby B.A.S., Thiruthaneeswaran N., Yang L., Pereira R.R., More E., McArt D.G., O'Reilly P., Bristow R.G., Williams K.J., Choudhury A., West C.M.L. Repurposing FDA approved drugs as radiosensitizers for treating hypoxic prostate cancer. *BMC Urol.* 2021;21(1):96.