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Original Article

Identification of key genes in breast cancer cell line under hypoxia condition: A bioinformatics analysis

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ABSTRACT

Introduction: The present study attempted to investigate the key pathways and genes which are associated with hypoxia in the human breast carcinoma cell line MDA-MB-231 with searched in gene expression omnibus (GEO) database for mRNA microarray data of MDA-MB-231 in normal and hypoxia condition.

Methods: Three GEO datasets GSE37340, GSE39042, and GSE42416 were downloaded from the Gene Expression Omnibus (GEO) database that these GEO profiles have of 9 cell lines in hypoxia condition and eight cell lines in normal condition. The differentially expressed genes (DEGs) between MDA-MB-231 cell line in hypoxia and normal condition were analyzed by Geo2R software. Next, all the differentially expressed genes (DEGs) with $p<0.05$ and fold change ≥ 1 or ≤ -1 was identified. Among all the differentially expressed genes, only 32 genes were at least in two datasets (31 up regulated and 1 down regulated) after gene integration. Moreover, DEGs ontology terms, Kyoto Encyclopedia and Genomes pathways were analyzed using EnrichR database. Subsequently, a protein-protein interaction network was constructed using STRING and MCODE software. Finally, the survival analyses performed with Kaplan Meier-plotter (KM) online dataset.

Results: Thirty-two genes were found to be at least two datasets (i.e., SLC2A3, BNIP3, ENO2, PFKFB3, PLOD2, SLC2A1, HK2, ADM, etc.) that two genes among up regulated genes (HK2, ADM) were expressed in all three datasets.

Conclusion: These identified genes and pathways could help to understand the mechanism of development of (Triple-negative breast cancer) TNBC under hypoxia condition. Also HK2, ADM, CENP family, might be promising targets for the TNBC treatment.

Introduction

One of the most common malignancies with the highest incidence and mortality rates in women is breast cancer (1). According to the molecular classification and microarray analysis of patient`s tumors, breast cancer is classified into five subgroups, including (2-4): i. Luminal A (Positive estrogen receptor, positive progesterone receptor, negative HER2 and low Ki67 and have the best prognosis); ii. Luminal B (positive estrogen receptor, positive progesterone receptor, HER2 positive, but also has low expression of estrogen receptor and HER2, and high Ki67); iii. HER2 over-expression (low or no-expression of estrogen receptor and progesterone receptor); iv. Basal-like (indicates a high genomic instability, positive EGFR, usually triple negative (ER-PR-1 / HER2-basal marker +) and has the shortest survival); v. Normal breast like (Low expression of estrogen receptor and progesterone receptor, HER2 negative, P53 positive and low Ki67).

Triple-negative breast cancer (TNBC) accounts for approximately 12% to 17% of all cases of breast cancer (5). Since the MDA-MB-231 cell line is a TNBC cell line, it`s biological characteristic and behaviour may be valuable(6).

Hypoxia is known as a very important pathological feature of solid tumors and has various effects on the biological behaviour of cancer cells (6). Therefore, adaptation to the hypoxic microenvironment is vital for a variety of pathological processes including survival, cell growth, neovascularization, metastasis and tumor sensitivity to treatment. (7, 8). Unfortunately, there is no sufficient information about the hypoxia role in breast cancer cells modulation (9).

We have used a bioinformatics microarray data analysis to identify the genes that are upregulated and downregulated by hypoxia in the hormone-independent MDA-MB-231 cell line. In the present study, we analyzed three gene expression profiles to obtain DEGs (differently expressed genes) in breast cancer cell line (MDA-MB-231 in a hypoxia and normoxia conditions. Functional enrichment and network analyses were applied to identify the DEGs and also describe the key genes as well as their potential molecular mechanisms in breast cancer. These upregulated and downregulated genes in this hormone-independent cell line could be a clue to studying hypoxia-related events and exploration of the novel therapeutic targets in human breast cancer. DEGs gained by at least two datasets were chosen for extra analysis. DEGs with fold change (|FC|)>1 and P<0.05 in all three datasets were chosen as biomarkers of the hypoxia effect on this cell line.

Methods

GEO database and Microarray data

The GEO database records numerous high-throughput functional genomic studies. These studies consist of data that are processed and normalized via several methods. We searched the GEO database (https://www.ncbi.nlm.nih.gov/geo/) for publicly available studies from January 2010 to October 2018 using the following keywords: “MDA-MB-231” AND “Hypoxia” (study keyword), “Humans” (organism), “Expression profiling by array” (study type) and 12 GEO series were identified. The inclusion criteria for studies were included (1) breast cancer cell line (MDA-MB-231) in hypoxia and normoxia condition, (2) gene expression profiling of mRNA, and (3) sufficient information to perform the analysis. As a result, three gene expression profiles (GSE37340, GSE39042, and GSE42416) were finally gained from the GEO database for analysis (Table1).

Data processing

Differentially expressed genes between a hypoxia and normoxia condition screening were applied by GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/)(12). P-value <0.05 was established as the cut off criteria for differentially expressed genes (DEGs). For supplementary analysis selected DEGs were achieved by at least two datasets. DEGs with p-value <0.05 and fold change (|FC|)>1 in all three datasets were selected as hypoxia effect biomarkers. Venn Diagrams were shaped by the software of Venn Diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/) to an exhibition the overlap of DEGs between the three datasets(13).

Functional and pathway enrichment analysis

Upregulated and downregulated genes were subjected to EnrichR (amp.pharm.mssm.edu/Enrichr/)(14) for three purposes: Gene Ontology(GO) assessment (15, 16), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (17) and function and pathway enrichment. Annotation, and chromosome location analyzed by bioDBnet (https://biodbnet-abcc.ncifcrf.gov/) as an online software (18).

Protein-protein interaction (PPI) network construction and analysis of modules

For the creation of a protein-protein interaction, integration used the STRING (Search Tool for the Retrieval of Interacting Genes) database (http://string-db.org/) as an online software (19). Moreover, the graphical analysis of networks of biomolecule interaction composed of protein, gene, and other types of interactions was evaluated by Cytoscape (version 3.6.1) (20). The DEGs were mapped to STRING to a PPI network construction and then visualized with Cytoscape. Then, the Molecular Complex Detection (MCODE) plug-in was used to screen modules of hub genes from the PPI network with cut-off degree equal to 10, the haircut on, node score cut-off = 0.2, k-core = 2, and max. depth = 100 (21).

Survival analysis

Survival Analysis was performed using datasets with term breast cancer (METABRIC, Nature 2012 & Nat Commun 2016) from the cBioPortal database (22), (survival analysis refers to the Overall Survival Kaplan-Meier Estimate). The datasets used in this study were composed of 2509 breast cancer samples/patients, and, then, they were filtered by a negative status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2). In total, 320 patients were selected that have TNBC status. Based on a relationship network built by MCODE, gene names were submitted in cBioPortal, and survival analysis was accomplished, of which those genes with Log rank Test p<0.05 were presented.

Results

Identification of DEGs

Three datasets GSE37340-GSE39042-GSE42416 were used that nine cell lines were in a state of hypoxia, and eight cell lines were in a state of normoxia. Totally 1241, 2562, 2081 genes, respectively were in the three datasets with significant expression changes (P-value less than 0.05). also 56, 150, 1737 genes upregulated with log fold change more than 1 with P-value less than 0.05 and 66, 98, 277 genes downregulated with log fold change less than -1 with P-value less than 0.05 respectively. Two genes, such as hexokinase 2 (HK2) and adrenomedullin (ADM), were reported as upregulated in the hypoxia condition in all three datasets. A total of 31 genes were reported to be upregulated in the hypoxia condition in two datasets and 1 gene (CENPN: Centromere protein N) was reported to be downregulated in the hypoxia condition in two datasets (Figure 1).

GO and pathway analysis

To assess the function of the DEGs, GO and KEGG pathway enrichment analyses were performed using DAVID and bioDBnet software. The upregulated genes involved in carbohydrate kinase activity, 6-phosphofructo-2-kinase activity, fructose-2,6-bisphosphate 2-phosphatase activity, phosphofructokinase activity, D-glucose transmembrane transporter activity, and hexose transmembrane transporter activity, while the downregulated gene involved in CENP-A containing nucleosome assembly, CENP-A containing chromatin organization and centromere complex assembly (Table 2). KEGG pathways for upregulated genes were ‘HIF-1 signaling pathway’,‘Central carbon metabolism in cancer’,‘Fructose and mannose metabolism’,‘Glycolysis / Gluconeogenesis’,‘Insulin signaling pathway’,‘Starch and sucrose metabolism’,‘Biosynthesis of antibiotics’ (Table 3).

Table 1. Three gene expression profiles and their features

	Platform	Sample count	In Hypoxia	In Normoxia	Year of publish	Ref
GSE39042	Affymetrix Human Genome U133 Plus 2.0	6	3	3	2012	(10)
GSE42416	Affymetrix Human Gene 1.0	3	2	1	2015	(11)
GSE37340	Affymetrix Human Gene 1.0	8	4	4	2015	(11)

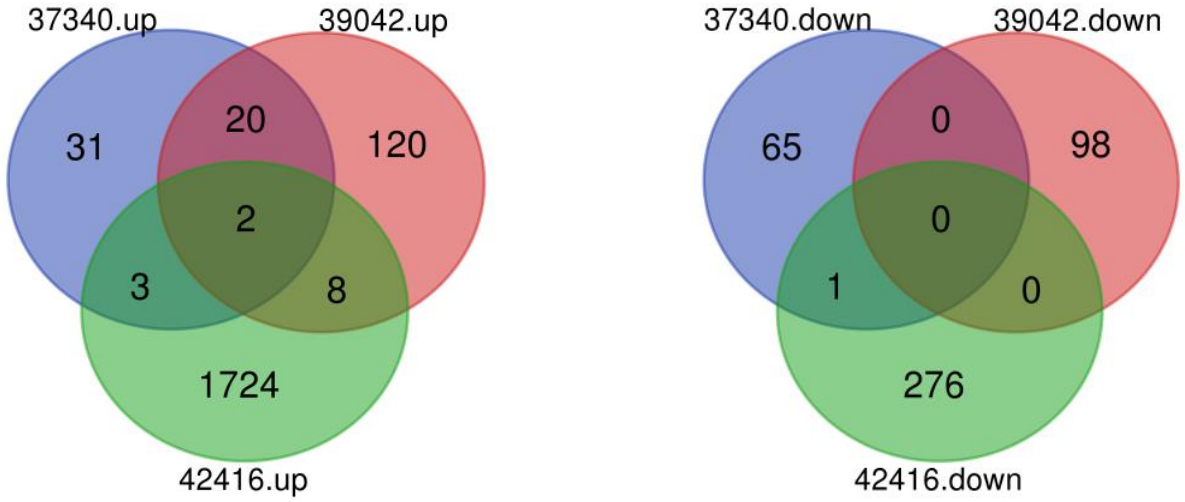

Figure 1. Upregulated and downregulated DEGs in MDA-MB-231 cell as a breast cancer cell line with hypoxia condition. Common DEGs among the three datasets were recognized via Venn diagrams. Two genes, including HK2 (hexokinase 2) and ADM (Adrenomedullin), were reported to be upregulated in the hypoxia condition in all three datasets. In addition to these two genes a total of 31 genes were reported to be upregulated in the hypoxia condition in two datasets and 1 gene were reported to be downregulated in the hypoxia condition in two datasets. (DEGs = differentially expressed genes)

Table 2. Top 3 GO terms enriched in DEGs in hypoxia status vs normal status

Upregulation PPI Network				
Pathway ID	**Pathway description**	**Gene count**	**False discovery rate**	**Matching proteins in your network (labels)**
GO.0019200	carbohydrate kinase activity	3	0.015	HK2,PFKFB3,PFKFB4
GO.0003873	6-phosphofructo-2-kinase activity	2	0.0311	PFKFB3,PFKFB4
GO.0004331	fructose-2,6-bisphosphate 2-phosphatase activity	2	0.0345	PFKFB3,PFKFB4
GO Molecular Function 2018 (EnrichR)				
	Name	**P-value**	**Adjusted p-value**	**Z-score**
(GO:0008443)	phosphofructokinase activity	0.003947	-3.08	29.30
(GO:0055056)	D-glucose transmembrane transporter activity	0.003947	-3.06	27.66
(GO:0015149)	hexose transmembrane transporter activity	0.005299	-2.78	23.21
***Down regulation* PPI Network**				
Pathway ID	**Pathway description**	**Gene count**	**False discovery rate**	**Matching proteins in your network (labels)**
GO.0034080	CENP-A containing nucleosome assembly	11	4.50E-27	CENPA,CENPH,CENPK,CENPL, CENPM,CENPN,CENPO,CENPQ, CENPT,ITGB3BP,MLF1IP
GO.0061641	CENP-A containing chromatin organization	11	4.50E-27	CENPA,CENPH,CENPK,CENPL, CENPM,CENPN,CENPO,CENPQ, CENPT,ITGB3BP,MLF1IP
GO.0034508	centromere complex assembly	10	2.19E-23	CENPA,CENPK,CENPL,CENPM, CENPN,CENPO,CENPQ,CENPT,I TGB3BP,MLF1IP

Table 3. KEGG pathways enriched in DEGs in hypoxia status vs normal status

Category	Term	Count	Pvalue	Genes	Fold Enrichment	Bonferroni	Benjamini	FDR
KEGG_PATHWAY	hsa04066:HIF-1 signaling pathway	6	2.80E-06	PDK1, MAP2K1, PFKFB3, SLC2A1, ENO2, HK2	23.50340136	2.60E-04	2.60E-04	0.003065
KEGG_PATHWAY	hsa05230:Central carbon metabolism in cancer	4	4.70E-04	PDK1, MAP2K1, SLC2A1, HK2	23.99305556	0.042755035	0.021611036	0.513046
KEGG_PATHWAY	hsa00051:Fructose and mannose metabolism	3	0.002706	PFKFB4, PFKFB3, HK2	35.98958333	0.222743553	0.080564127	2.922677
KEGG_PATHWAY	hsa00010:Glycolysis / Gluconeogenesis	3	0.011468	LDHA, ENO2, HK2	17.18905473	0.657895866	0.235214614	11.86194
KEGG_PATHWAY	hsa04910:Insulin signaling pathway	3	0.044273	PPP1R3B, MAP2K1, HK2	8.345410628	0.985172509	0.569261654	39.08736
KEGG_PATHWAY	hsa00500:Starch and sucrose metabolism	2	0.078245	GBE1, HK2	23.26599327	0.999488031	0.717160112	59.01461
KEGG_PATHWAY	hsa01130:Biosynthesis of antibiotics	3	0.094247	LDHA, ENO2, HK2	5.432389937	0.999899561	0.731562544	66.16514

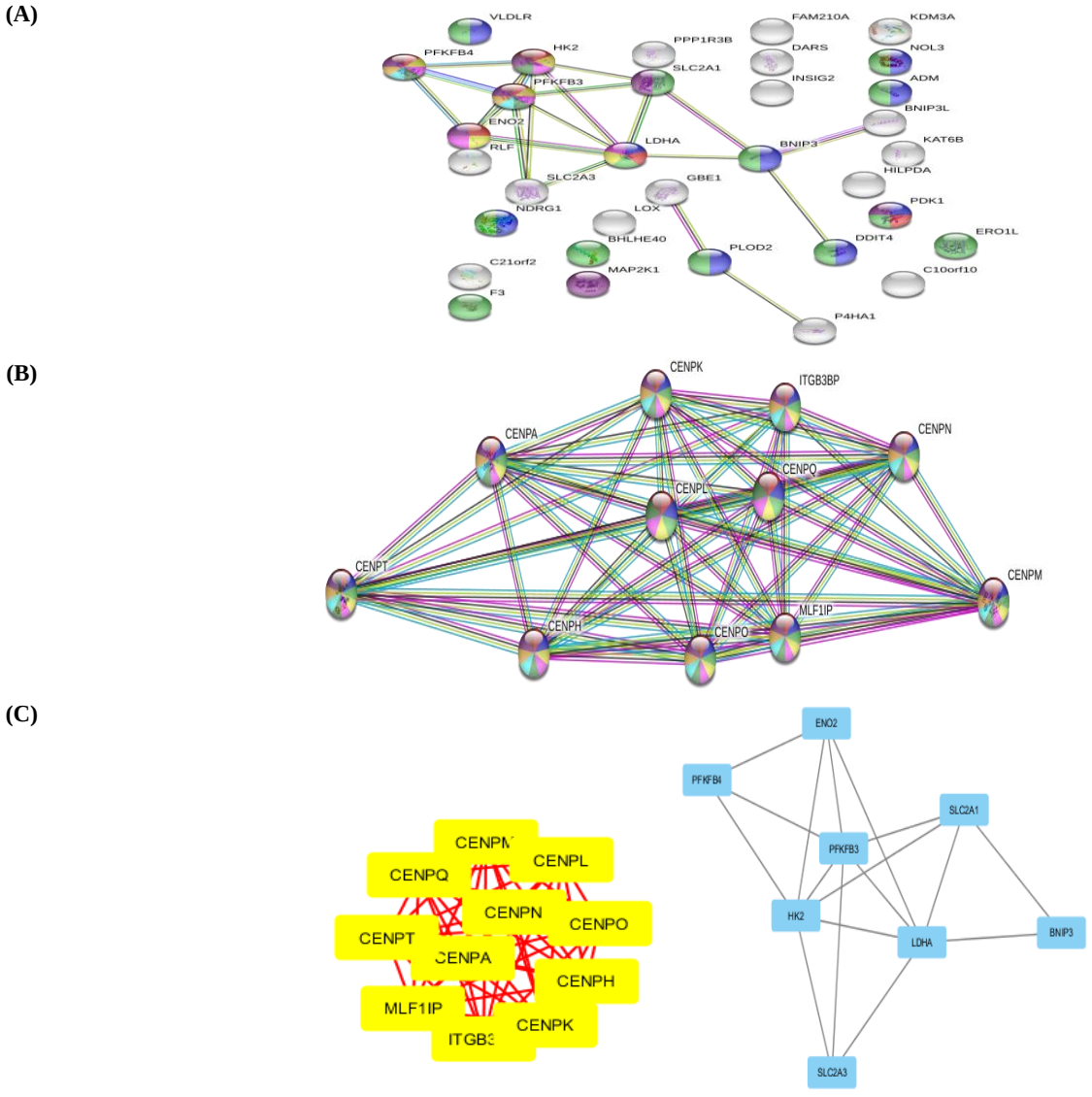

Figure 2. PPI networks of DEGs identified in MDA-MB-231 in hypoxia status versus normal status. A: upregulation of DEGs identified in at least two datasets, were used to construct the PPI network. The lines between nodes represent the interactions between genes. B: downregulation of DEG identified in at least two datasets, were used to construct the PPI network. C: Two PPI modules were extracted from the PPI network using MCODE in Cytoscape. PPI: protein-protein interaction; DEGs: differentially expressed genes; blue nodes indicate upregulated genes; yellow nodes indicate downregulated genes

PPI network construction

Upregulated DEGs in hypoxia condition were mapped with the STRING database. With a PPI score >0.4, a PPI network of upregulating genes with 33 nodes and 21 edges (Figure 2A) and downregulate gene with 11 nodes and 55 edges was constructed (Figure 2B). One module was obtained from a PPI network of DEGs using MCODE, upregulate with 8 nodes and 17 edges, and downregulate with 11 nodes and 55 edges (Figure 2C).

Survival Analysis

All the DEGs underwent survival analysis using cBioPortal datasets with term of breast cancer (METABRIC, Nature 2012 & Nat Commun 2016). 320 TNBCs were selected from 2509 breast cancer samples/patients by filtering by a negative status of ER/PR/Her2. Among all the DEGs, CENPL (Centromere Protein L) significantly shortened the life expectancy ($p<0.05$) (Figure 3). Oncoprint showed that 41 altered in 320 sequenced cases/patients, as shown in Table 4 and Figure 4.

Table 4. Median Months Survival in patients with altered CENPL expression

	Number of Cases, Total	Number of Cases, Deceased	Median Months Survival
Cases with Alteration(s) in Query Gene(s)	41	27	68.13333333
Cases without Alteration(s) in Query Gene(s)	279	141	176.1

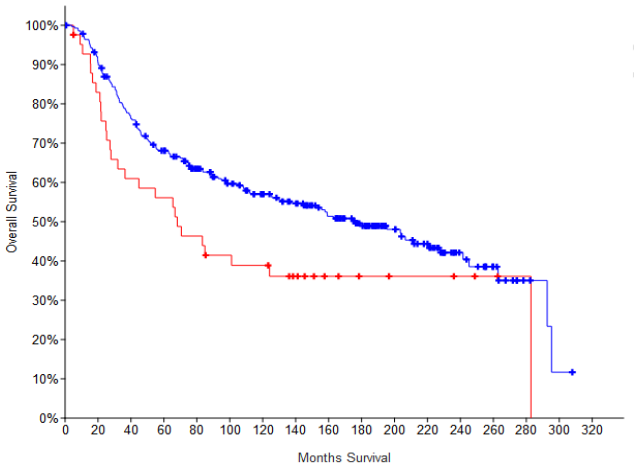

Figure 3. Overall Survival Kaplan-Meier estimate of all DEGs in 320 TNBC from TCGA datasets with term of breast cancer (METABRIC, Nature2012 & Nat Commun 2016). Red line represents cases with alterations. Blue line represents cases without alterations (CENPL, P=0.0241)

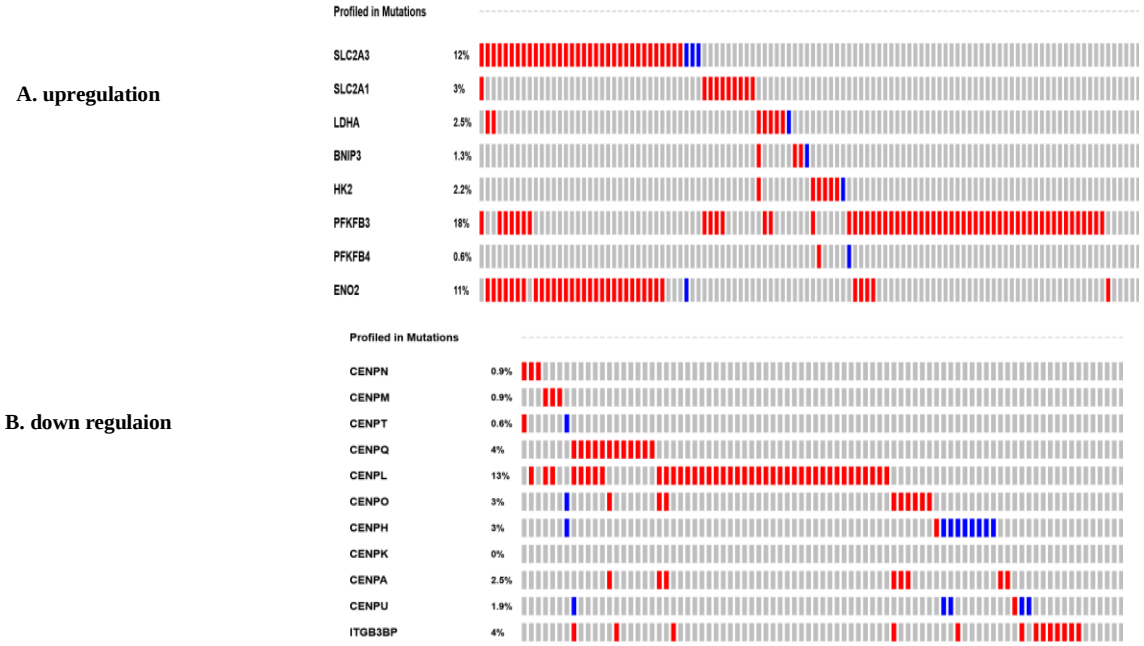

Figure 4. Oncoprint of genes. Every column represents a sample/patient. Red represents amplifcation; blue represents deep deletion and gray represents no alteration

Discussion

Hypoxia can alter metabolic pathways and promote cell survival by adapting to the local microenvironment. It can also regulate the receptor tyrosine kinases activity and activate multiple signaling pathways that lead to tumor cell proliferation, survival, metastasis and induce epithelial-mesenchymal transition (EMT) and facilitate tumor metastasis (23, 24). From the therapeutic point of view, several data showed that oxygen concentration alters the sensitivity of chemotherapeutic agents (25). Therefore, it would be beneficial to characterize and identify the genes regulated by hypoxia in cancer (9).

In the present study, we investigated the hypoxia effects on the human breast carcinoma cell line MDA-MB-231. We demonstrated that hypoxia influence different pathways in MDA-MB-231 cells. The solid tumor's growth result in the creation of a hypoxic microenvironment in the internal region of the tumor due to insufficient oxygen diffusion. Adaptation with hypoxia has an advantage for increasing of chemoresistance and metastatic ability (26, 27).

In our study, 32 genes were found to be at least in two datasets including SLC2A3, BNIP3, ENO2, PFKFB3, PLOD2, SLC2A1, INSIG2, DDIT4, NDRG1, GBE1, ERO1A, BHLHE40, FAM210A, P4HA1, LOX, PDK1, BNIP3L, PFKFB4, KDM3A, DARS, LDHA, MAP2K1, F3, VLDLR, PPP1R3B, C10orf10, C21orf2, HILPDA, KAT6B, RLF,NOL3, HK2 , ADM, CENPN) that two genes were expressed in all three datasets (HK2 , ADM) and one gene was downregulated in two datasets (CENPN).

HK2 phosphorylates glucose to produce glucose-6-phosphate, thus making deal glucose to the glycolytic pathway. This isoenzyme is commonly found in skeletal muscle and is placed in the outer membrane of the mitochondria (28). The expression of HK2 has a role in response to insulin. Rat studies suggested that it is involved in the glycolysis increase or elevation that leads to a rapid increase in the cancer cells growth (9). Studies of co-expression showed p53 overexpression significantly activates the hexokinase 2 promoters(29). HK2 is one of the four isoforms of the hexokinases family, denoted as HK I to IV in mammalian tissues (30). It's overexpression in several human carcinomas (31-33), suggests that HKII plays an important role in supplying energy to cancer cells.

Additionally, in the present study, the expression of ADM, Adrenomedullin (ADM), was increased in all datasets. ADM is a peptide that was originally isolated in 1993 from human pheochromocytoma tissues and consists of 52 amino acids(34). It`s functions as a local paracrine and autocrine mediator are multiple biological activities such as cell growth, vasodilatation, hormone secretion regulation, natriuresis, and antimicrobial effects (35-38). High production of ADM has been established in the human adrenal medulla, heart, lung, kidney, and pancreas and pheochromocytoma tissues(39). Expression of ADM has been reported in different human cancers such as pulmonary, adrenocortical, choroid plexus, colorectal, ovarian, prostate, trophoblastic, endometrial, breast, kidney, and larynx cancer(40). Overexpression of ADM in breast cancer cells has been seen in hypoxia condition that is associated with high levels of proteins involved in oncogenic signal transduction pathways (i.e., Ras, Raf, PKC, and MAPKp49) and lower levels of pro-apoptotic proteins (such as Bax, Bid, and caspase 8)(41).

Centromeres play a very important role in regulating the kinetochore formation and segregation of the chromosomes in cell division. Defects in these activities cause aneuploidy, neoplastic changes, chromosomal instability and tumorigenesis. Centromeres contain various proteins such as: CENPC,CENPH/CENPI/CENPK, CENPL/CENPM/CENPN, CENPO/CENPP/CENPQ/CENPR/ CENPU, CENPT/CENPW, CENPS/CENPX and CENP-A, CENP-B, CENP-E, CENP-I that have different functions, and in some cancers they are associated with invasion and have been introduced as cancer biomarkers (42, 43).

CENPN is one of the important proteins of this family that it`s C-terminal is connected to the CENPL and it`s N-terminal interacts with the CENPA. The CENPL and CENPN complex interact with the CENPC and CENPH-K-I-M complex (44). CENPN is also attached to the kinetochores in the G2 and S phases, but this protein is separated from kinetochores during M and G1 phases (45). CENPL is a necessary protein for microtubule stability. The defect in CENPL interferes with the chromosome movement regulation and the chromosome disjunction in the prometaphase (46). CENPL has a significant P-value < 0.05 in survival Kaplan-Meier and Spearman and Pearson Correlation: 0.463, P-Value: 2.25e-15 with CENPN in cBioPortal database, also in PPI enrichment has P-value :< 1.0e-16 in networks.

Therefore, according to our research data analysis, HK2, ADM and CENPL can be recommended as a potential biomarker and therapeutic target of hypoxia status in triple negative breast cancer.

Conclusion

The present study demonstrated that triple-negative breast cancer under hypoxia condition exhibits high HIF-1 signaling pathway, glycolysis / gluconeogenesis, central carbon metabolism in cancer, insulin signaling pathway, low nucleosome assembly, chromatin remodeling at the centromere and chromatin organization pathways. Also HK2, ADM, CENP family, might be promising targets for the TNBC treatment. However, one of the limitations of this study was the absence of experimental confirmation. In the future, these predicted results can be proved by experiments methods such as qRT-PCR and Western Blot.

Ethical disclosure

Not applicable.

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Nothing to declare.

Author contributions

All authors contributed equally in this manuscript.

Conflict of interest

No conflict of interest has been declared by the authors.

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