GENE EXPRESSION PROFILING AND CLINICAL RELEVANCE TO UNDERSTAND THE ROLE OF HYPOXIA AND IMMUNE SIGNALING GENES AND PATHWAYS IN BREAST CANCER

Mohammad Azhar Kamal[1,2,#], Mohiuddin Khan Warsi[1,2,#], Afnan Alnajeebi[1], Haytham A Ali[1,3], Nawal Helmi[4,5], Mohammad Asrar Izhari[6], Saad Mustafa[7], and Mohammad Mobashir[8,*]

Author information: [#]Shared first author, [1]University of Jeddah, college of science, department of biochemistry, Jeddah, the Kingdom of Saudi Arabia, [2]University of Jeddah, Centre for Science and Medical Research (UJ-CSMR), Jeddah, the Kingdom of Saudi Arabia, [3]Department of Biochemistry, Faculty of Vet. Medicine, Zagazig University, Zagazig, Egypt, [4]Department of Biochemistry, College of Sciences, University of Jeddah, Jeddah, , the Kingdom of Saudi Arabia, [5]Department of Medical Laboratory Technology College of Applied Medical Sciences, University of Jeddah, [6]Faculty of Applied Medical Sciences, University of Al-Baha, Al-Baha, the Kingdom of Saudi Arabia, [7]School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India Pin code-110067, [8]Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institute, Novels väg 16, 17165 Solna, Stockholm, Sweden, Tel: +46 70 872 3675, m.mobashir@cdslifesciences.com

Abstract: Hypoxia often occurs in cancer and helps the cells in adapting different responses than normal cells, such as the activation of signaling pathways which regulate proliferation, angiogenesis, and cell death. Moreover, there are a number of genes that are known to be associated with these mentioned processes and functions. In this study, our goal was to understand the impact of alteration in the expression of hypoxia and immune system-related genes and their contribution to breast cancer. For this purpose, we have collected the hypoxia-associated genes based on the literature related to diverse biological processes and functions. For all these genes, we have studied the survival analysis, breast cancer gene expression profiling, and relevant hypoxic gene alterations. Based on our study, we concluded that there are 17 critical pathways and 40 genes from the hypoxic gene list that appear to play major roles in the case of breast cancer and overall, we observed that immune signaling pathways and its components are highly altered in cases of breast cancer. Among the top ranked hallmarks of molecular signatures are apoptosis, hypoxia, DNA repair, E2F targets, MYC targets, androgen and estrogen response, and TNFa signaling.

Keywords: Hypoxic genes; immune signaling pathways; breast cancer; gene expression profiling; survival analysis; inferred functions, hallmarks of molecular signatures.

INTRODUCTION Almost all cancers are known to be highly heterogeneous in nature and breast cancer is also one of them which has many clinical outcomes and for this cancer initial surgery, adjuvant chemotherapy, and endocrine therapy are available as therapeutic options. However, breast cancer patients still have a risk of recurrence [1-4]. Keeping in the views of its nature of heterogeneity and complexity of therapeutic intervention, the promising field of biomarker research mainly focuses on the identification of factors predicting long-term relapse-free survival in breast cancer survivors. Clinical evidence from previous studies has demonstrated that tumor hypoxia may play an important role in breast cancer and these hypoxic tumors are related to poor prognosis and survival [5-7]. When the availability of intravascular oxygen content in tumors becomes lower than the metabolic requirements of cancer cells, a hypoxic condition arises. Several families of transcription factors called hypoxia-inducible factors (HIFs) are adapted by hypoxic tumor cells. The first member of this transcription factor family, HIF-1 which is universally expressed and under normoxic environments it is hydroxylated by propyl-hydroxylases (PHD) [8], which ultimately leads to its degradation. Alternatively, in hypoxic conditions, it is not degraded and instead translocates into the nucleus and where binds with the subunit HIF-1 beta and the

*Corresponding author: Mohammad Mobashir[8]Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institute, Novels väg 16, 17165 Solna, Stockholm, Sweden, Tel: +46 70 872 3675, m.mobashir@cdslifesciences.com

transcriptional coactivator p300. This complex regulates the expression of multiple genes through hypoxia response elements (HRE) [9,10]. Previously, it was demonstrated that dysfunctions of the PI3K/AKT and RAS/MAPK signaling pathways lead to the regulation of HIF-1 alpha and genetic alterations are also occurring such as activation of oncogenes (*HER2*) and/or loss of tumor suppressor genes (*VHL* or *PTEN*). It is also known that hypoxia-related genes play critical roles in tumor progression because these genes are involved in several cellular processes which include cell differentiation, survival, angiogenesis, migration, and metastasis. A similar study showed that HER2+ breast cancer cells harbored high amplification of HER genes compared to HER2- breast cancer cells [11]. The observed overexpression of *NHERF1* led to the progression of high-grade tumors and increased expression of HIF-1 alpha protein in hypoxic breast cancer cells. PHD2 encodes the dioxygenase and catalyzes the post-translational hydroxylation of HIF-1 alpha protein under normoxia. During hypoxic conditions, PHD2 expression is enhanced, thus increased levels of PHD2 are associated with relapse and tumor metastasis. Similarly, the expression of PGK1 was also observed to be enhanced in hypoxic breast cancer conditions. During metabolism, these tumor cells significantly expressed six genes, *PGK1*, *LDHA*, *TPI*, *ENO1*, *EPO,* and *ETS1*. These markers were overexpressed in the relapse group compared with the non-relapse group because these genes are involved in glucose metabolism and affect the implication of HIF factors in glucose metabolism of tumor cells. In hypoxic conditions, cancer cells redirect their aerobic metabolism to anaerobic metabolism by activating glycolysis, which becomes the main source of energy. Expression of lactate dehydrogenase A (LDHA) is increased leading to increased ATP production, cell proliferation and conversion of pyruvate into lactate under hypoxia. The lactate is absorbed and used as a respiratory substrate for promoting angiogenesis and metastasis and regulates the HRE elements. Moreover, HRE elements are also identified in the promoter of EST1 and are involved in its transcriptional activation under hypoxic conditions and increase the risk of invasive breast cancer.

The gene expression profiling by microarray is a powerful tool for cancer biomarker discovery and facilitates the correlation of expression profiles with clinical outcomes in both prospective and retrospective studies. Previously, a molecular signature specific to hypoxia responses in breast cancer was defined and was associated with tumor aggressiveness and the risk of recurrence.

The identification of global expression analysis of multiple genes and pathways might conquer most of the limitations of current markers and other detection methods responsive to hypoxia. The gene expression analysis of hypoxia genes also has the potential to imitate the complexity of the tumor. Thus, expression analysis can be used to reveal the nature of the hypoxic response to a specific therapy in terms of gene networks and therefore, may be helpful to improve our understanding of mechanisms of resistance and may provide potential value of the risk score of relapse following specific therapies.

Our study is distinctive from others, as we collected the hypoxia and immune system-related genes associated with diverse biological processes and hallmarks of molecular signatures (MsigDB [5,12-17]. For all these genes, we have studied the survival analysis. In this analysis, we have considered the genes induced by hypoxia for these categories: oxygen transport and iron metabolism, angiogenesis, glycolysis, and glucose uptake, transcription factors, metabolism/pH/neurotransmitters, growth factors and cytokines, stress-response pathways, cell adhesion, ECM, cytoskeleton, and proteases/coagulation.

In addition to investigating the survival analysis, gene expression profiling and relevant hypoxia gene alterations in breast cancer were also studied. Based on our study, we concluded that there are 17 critical pathways and 40 genes which appear to play major roles in breast cancer in terms of hypoxia and its association. We also concluded that immune signaling pathways and its components are highly altered in cases of breast cancer followed by their hallmarks of molecular signatures.

Methods: For this study, the dataset used for gene expression is GSE42568 for breast cancer which we have obtained from GEO (Gene Expression Omnibus) where the dataset contains normal samples (17) and tumor samples (104). These gene expression profiling datasets were generated from the Affymetrix Human Genome U133 Plus 2.0 Array. This dataset consists of 104 breast cancer biopsies (removed prior to any treatment with tamoxifen or chemotherapeutic agents) from patients aged between 31 years and 89 years at the time of diagnosis (mean age = 58 years). Twenty were less than 50 years old and seventy-seven women were 50 years or older at diagnosis. For this work, we classified samples as either tumor or normal samples irrespective of the age, treatment/therapy, or duration of treatment [18].

For differential gene expression analysis, we have compared the tumor samples with normal samples to generate the DEGs list. In short, the basic steps involved are raw file (.CEL) processing, intensity calculation, and normalization. For normalization [25-27], GCRMA [25,28-31]. RMA, and EB are the most commonly used approaches. Here, we have used EB for raw intensity normalization. After normalization, we analyzed gene expression patterns [25,26, 32-36] and its inferred functions [34,35].

For differential gene expression prediction and statistical analysis, MATLAB functions (e.g., mattest) was used. For pathway analysis, we used KEGG [38] database and had our code designed to the pathway and the network analysis.

For generating DEGs network, FunCoup2.0 [39] was used for all the networks throughout the work and cytoscape [40] was used for network visualization. For most of our coding and calculations, MATLAB was used. FunCoup2.0 predicts four different classes of functional coupling or associations, such as protein complexes, protein-protein physical interactions, metabolic, and signaling pathways [41]. For hallmarks of molecular signatures analysis, we used MSigDB v7 [16].

RESULTS

Hypoxic genes and gene expression profiling reveal critical genes and the pathways associated with breast cancer

We studied the gene expression profiling by using the publicly available dataset from gene expression omnibus (GEO) GSE4256 [18]. This dataset contained 17 normal samples and 104 tumor samples (total human samples=121). We performed differential gene expression profiling and functional annotation for the differentially expressed genes (DEGs) and compared them with the list of hypoxic genes. We observed that there are 40 hypoxic genes (out of 185 genes) which are differentially expressed in the case of breast cancer (Figure 1a). Since we had a large number of DEGs (4664 DEGs) at +/- 2.0 fold change, we grouped the list of genes up to +/- 7.0 fold changes. We observed that even at +/- 7.0 fold change the number of DEGs was 376 and similarly, in the case of pathway enrichment analysis, we saw that the number of enriched pathways has been reduced from 142 to 17 (Figure 1b and 1c). Based on this result, we concluded that there are a large number of hypoxic genes that are associated with breast cancer and irrespective of the fold change, the set of pathways is always enriched or altered in cases of breast cancer.

Seventeen pathways are dominantly altered as a result of breast cancer development

As mentioned above, 17 pathways are always enriched or altered in the case of breast cancer (Figure 2a). Since the p-values were infinitesimally small for many pathways at different fold changes, we converted the p-values into -10*log10(p-values). The p-values may be interpreted by color, as blue represents ≥0.05 and red represents p-values ≤1e-22.

In addition, we used a network database to map out the links between the hypoxic genes which are differentially expressed for of breast cancer samples. We observed that XRCC6, CDK2, CDK6, PRKCA, ENO1, ACAT1, CDKN1A, and HYOU1 are the genes which show very high connectivity. Furthermore, we noted that there are several genes which are not connected with any gene (Figure 2b).

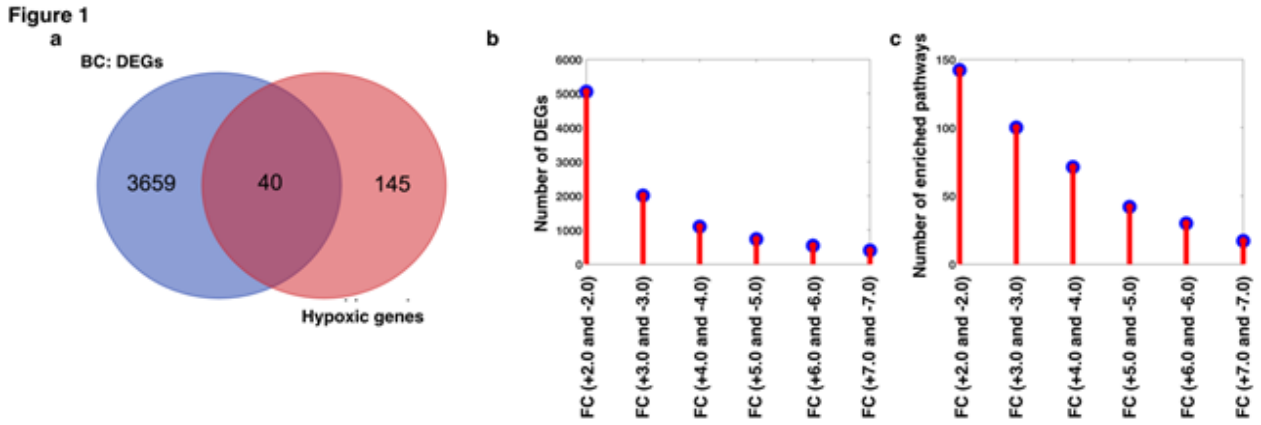

Figure 1: Hypoxic genes and gene expression profiling. (a) Venn diagram represents the common genes between the list of hypoxic genes and the DEGs. Here, the overall DEGs have been compared to the list of hypoxic genes collected from previous works (b) Number of DEGs at six different fold changes (from +/- 2 to +/- 7), and (c) the number of pathways at six different fold changes (from +/- 2 to +/- 7).

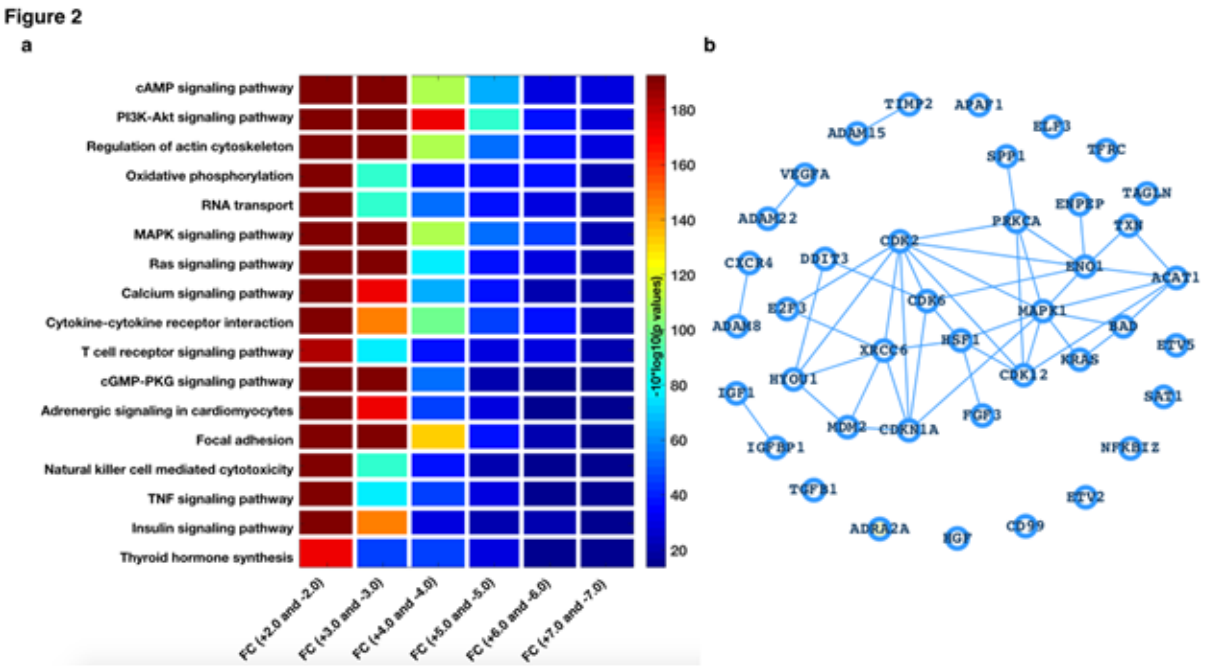

Figure 2: Pathways enriched as a result of differential gene expression profiling. (a) The 17 enriched pathways are enriched for all the six ranges of fold changes. (b) The network of hypoxic DEGs. The list hypoxic genes differentially expressed.

Differentially expressed hypoxic genes and their role in breast cancer

In the previous work, role of hypoxic genes have been explored and discussed in cancer [19,20]. Figure 1a represents the common genes between hypoxia and the DEGs. Further, we analyzed the gene expression pattern of these genes in normal samples and breast cancer tumor samples (Figure 3a). We noted that there is a significant difference in the expression pattern of hypoxic genes in normal samples compared to the tumor samples. As far as the clinical relevance is concerned, we found that only three genes (CD99, SAT1, and ADAM22) do not have significant p-values in the case of survival analysis in terms of overexpression (Figure 3b).

Immune signaling pathways are dominantly affected in case of breast cancer with respect to all other enriched pathways

For understanding the role of immune signaling pathways, we analyzed the genes which belong to the 17 enriched pathways shown previously (377 for FC > 7.0 and FC < -7.0). 312 genes belong to the following: cytokine-cytokine receptor interaction, T-cell receptor signaling pathways, cGMP-PKG signaling pathways, Natural killer cell-mediated cytotoxicity, and TNF signaling pathways (Figure 4), while there are only 39 genes which belong to the remaining pathways (Figure 2a).From these 39 genes, there are a number of genes that are common to these pathways. Therefore, based on this analysis, we concluded that immune signaling pathways and their components are highly altered in cases of breast cancer.

We have also presented a list of the hallmarks of molecular signatures in Table 1 where there are a large number of molecular signatures including hypoxia, apoptosis, cell cycle, and immune signaling components and their p-values are extremely low (5.42E-20) leading to its high significance. Based on this study, we concluded that both the KEGG enriched pathways and enriched hallmarks of molecular signatures are of extreme significance in terms of clinical perspective.

DISCUSSION Using the publicly available dataset, we investigated the gene expression profile for breast cancer. The current literature has focused on selected genes and pathways or provided a generalized view. Here we have investigated the list of hypoxic genes, critical pathways, and the genes which appear to be clinically highly significant in breast cancer. *CXCR4, IGF1, HYOU1, ADRA2A, ETV2, NFKBIZ, SPP1, VEGFA, TGFB1, MAPK1, MDM2, ENPEP, CDK2, ENO1* are among the top-ranked genes which appeared highly significant in terms of patient survival and were associated with hypoxia in breast cancer. Our work may aid in diagnosing breast cancer

Table 1: Enriched hallmarks signatures and their p-values

HALLMARK_ADIPOGENESIS	5.42E-20
HALLMARK_ALLOGRAFT_REJECTION	5.42E-20
HALLMARK_ANDROGEN_RESPONSE	5.42E-20
HALLMARK_APICAL_JUNCTION	5.42E-20
HALLMARK_APOPTOSIS	5.42E-20
HALLMARK_COAGULATION	5.42E-20
HALLMARK_COMPLEMENT	5.42E-20
HALLMARK_DNA_REPAIR	5.42E-20
HALLMARK_E2F_TARGETS	5.42E-20
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	5.42E-20
HALLMARK_ESTROGEN_RESPONSE_LATE	5.42E-20
HALLMARK_G2M_CHECKPOINT	5.42E-20
HALLMARK_GLYCOLYSIS	5.42E-20
HALLMARK_HEME_METABOLISM	5.42E-20
HALLMARK_HYPOXIA	5.42E-20
HALLMARK_IL2_STAT5_SIGNALING	5.42E-20
HALLMARK_INFLAMMATORY_RESPONSE	5.42E-20
HALLMARK_INTERFERON_GAMMA_RESPONSE	5.42E-20
HALLMARK_KRAS_SIGNALING_DN	5.42E-20
HALLMARK_KRAS_SIGNALING_UP	5.42E-20
HALLMARK_MITOTIC_SPINDLE	5.42E-20
HALLMARK_MTORC1_SIGNALING	5.42E-20
HALLMARK_MYC_TARGETS_V1	5.42E-20
HALLMARK_MYOGENESIS	5.42E-20
HALLMARK_OXIDATIVE_PHOSPHORYLATION	5.42E-20
HALLMARK_P53_PATHWAY	5.42E-20
HALLMARK_PI3K_AKT_MTOR_SIGNALING	5.42E-20
HALLMARK_PROTEIN_SECRETION	5.42E-20
HALLMARK_SPERMATOGENESIS	5.42E-20
HALLMARK_TNFA_SIGNALING_VIA_NFKB	5.42E-20
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	5.42E-20
HALLMARK_UV_RESPONSE_DN	5.42E-20
HALLMARK_UV_RESPONSE_UP	5.42E-20
HALLMARK_XENOBIOTIC_METABOLISM	5.42E-20
HALLMARK_FATTY_ACID_METABOLISM	1.56E-16
HALLMARK_IL6_JAK_STAT3_SIGNALING	2.81E-15
HALLMARK_PEROXISOME	7.65E-13
HALLMARK_BILE_ACID_METABOLISM	1.15E-11

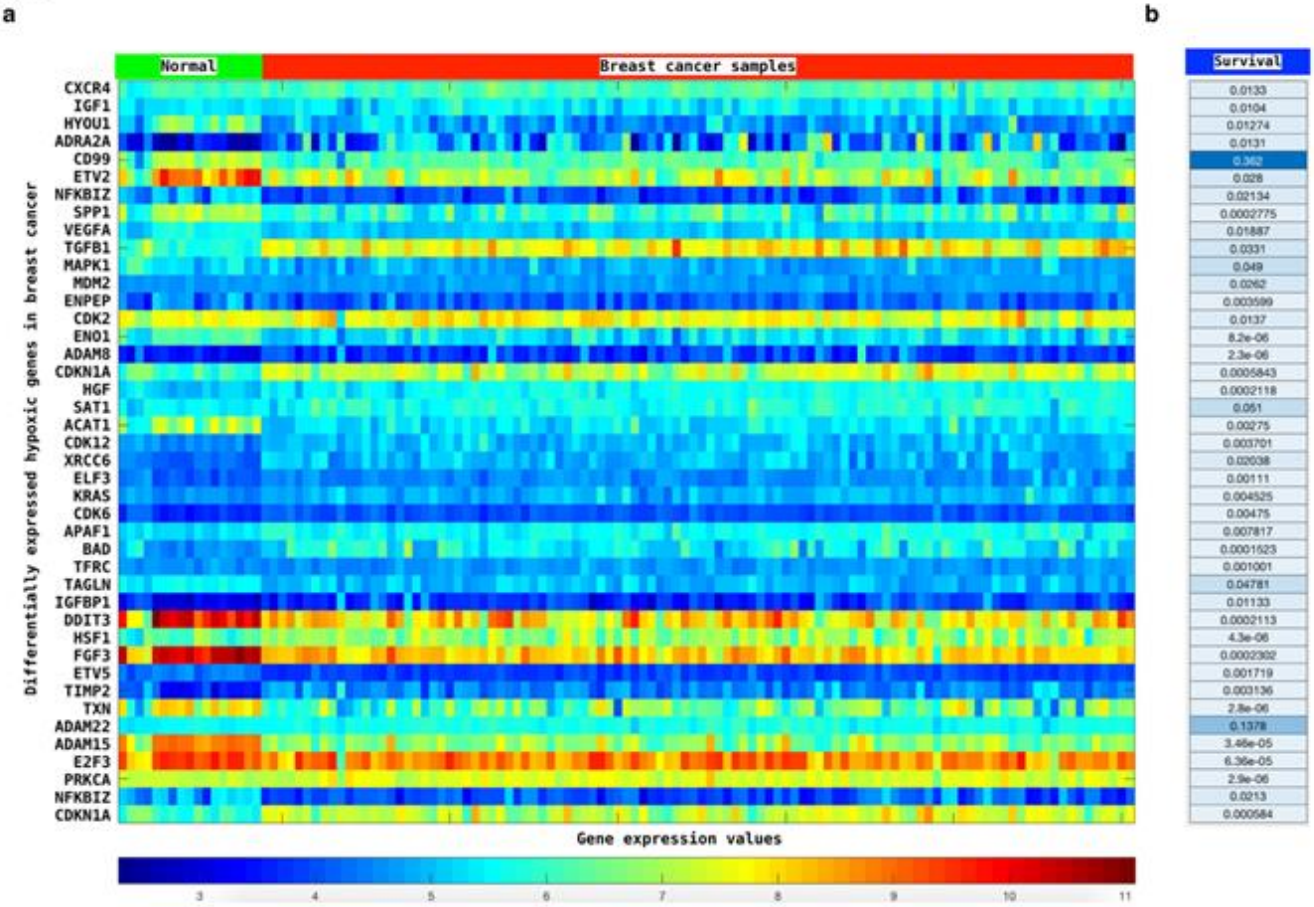

Figure 3: Differentially expressed hypoxic genes in breast cancer. (a) The genes which are differentially expressed and are associated with hypoxia have been presented here with their respective expression values in both normal and tumor samples. (b) Clinical significance of the hypoxic genes after survival analysis in terms of its overexpression.

patients, as we have presented the differentially expressed genes, their inferred pathways, and the clinical impact of the selective hypoxic genes. Furthermore, we noted that immune signaling pathways and their components are highly altered in breast cancer, which also supports that the immune system is a major therapeutic target point [21-24].

Hypoxia often occurs in cancer and such occurrence allows the cells to adapt to different responses compared to normal cells, such as the activation of signaling pathways which regulate proliferation, angiogenesis, and cell death. There are a large number of genes that are known to be associated with diverse biological processes and their control and coordination in hypoxia response differs between cancer types. In this study, our goal was to understand the impact of alteration of the expression of hypoxia-related genes and survival in breast cancer.
It is known that breast cancer is a heterogeneous disease that has many clinical outcomes in which therapeutic options such as initial surgery, adjuvant chemotherapy, and endocrine therapy are available, but breast cancer patients still have a risk of relapse. An important, promising field is biomarker research or discovery (which means identification of factors predicting long-term relapse-free survival in breast cancer survivors). As mentioned, there are many molecular signatures including hypoxia, apoptosis, cell cycle, and immune signaling components that are highly significant. Based on this study, we concluded that both the KEGG enriched pathways and enriched hallmarks of molecular signatures are extremely significant in terms of clinical perspective.

The combined study of hypoxic genes and the gene expression profiling in breast cancer may help in terms of diagnostic purpose and clinical relevance. Here, we have not only analyzed the hypoxic genes but also the overall gene expression profile and the major pathways which are enriched at a very high threshold of fold changes. These pathways are cytokine signaling, TNF signaling, NK cell-

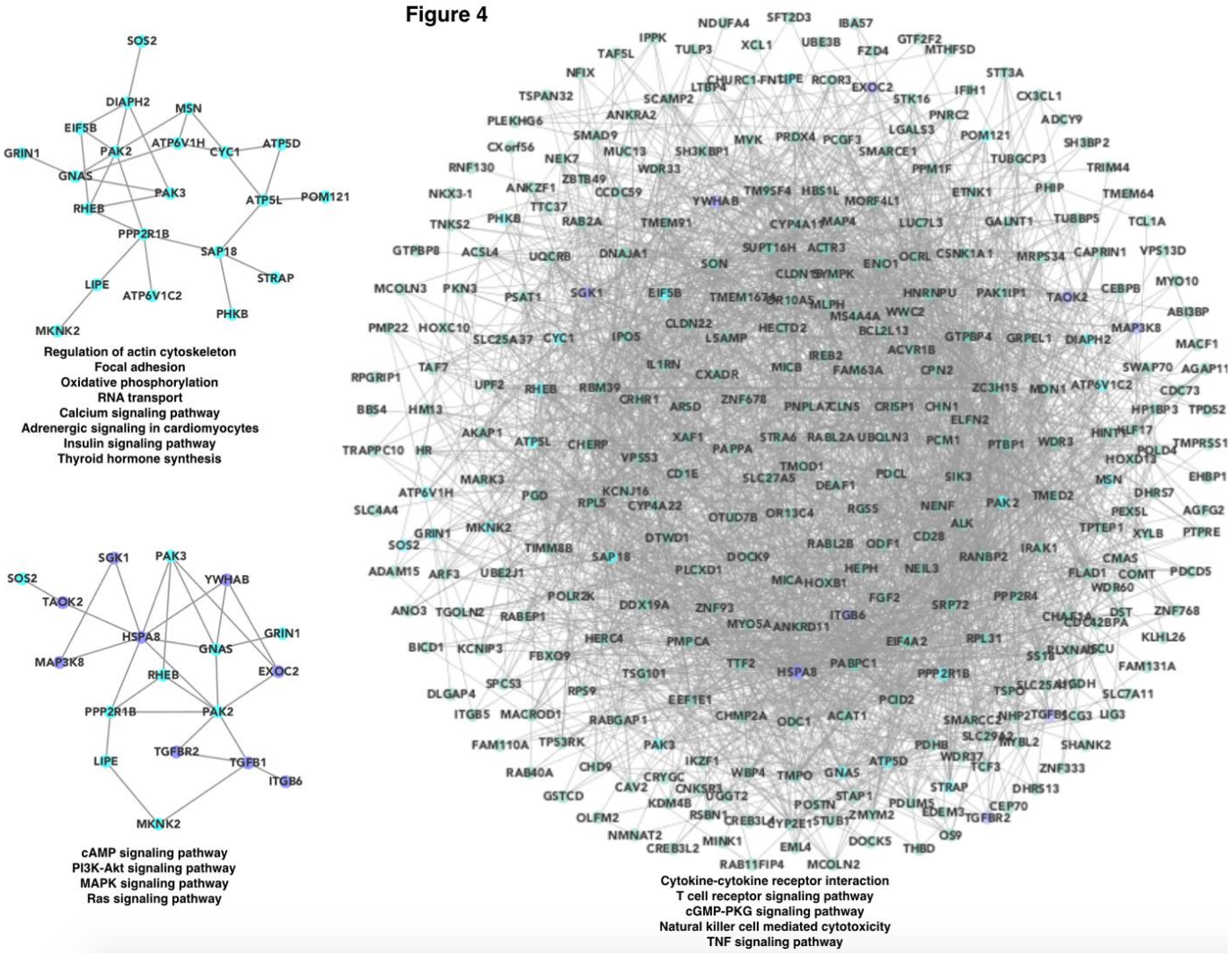

Figure 4: Immune signaling pathways are dominantly enriched. Here, the top enriched pathways have been placed into groups and for each group the network, has been mapped out.

mediated cytotoxicity, adrenergic signaling, AKT, MAPK, oxidative phosphorylation, and more. These 17 pathways (Figure 2a) not only are known to be associated with cancer but also other diseases including infection and inflammation.

CONCLUSION For all hypoxic differentially expressed genes, we have studied the survival analysis, gene expression profiling, and relevant hypoxic gene alterations in breast cancer. Based on our study, we conclude that there are 17 critical pathways and 40 genes which appear to play major roles in breast cancer associated with hypoxia and immune signaling pathways and their components appear to be the leading source of aberration in terms of gene expression.

REFERENCES

1. Wyatt AW, Mo F, Wang K, et al. Heterogeneity in the inter-tumor transcriptome of high-risk prostate cancer. August 2014:1–14. doi:10.1186/s13059-014-0426-y.
2. Liu AY, Roudier MP, True LD. Heterogeneity in Primary and Metastatic Prostate Cancer as Defined by Cell Surface CD Profile. *The American Journal of Pathology*. 2010;165(5):1543–1556. doi:10.1016/S0002-9440(10)63412-8.
3. Hu J, Locasale JW, Bielas JH, et al. Heterogeneity of tumor-induced gene expression changes in the human metabolic network. *Nat Biotechnol*. 2013;31(6):522–529. doi:10.1038/nbt.2530.
4. Chapman A, del Ama LF, Ferguson J, Kamarashev J, Wellbrock C, Hurlstone A. Heterogeneous Tumor

Subpopulations Cooperate to Drive Invasion. *CellReports*. 2014;8(3):688–695. doi:10.1016/j.celrep.2014.06.045.
5. Harris AL. HYPOXIA — A KEY REGULATORY FACTOR IN TUMOUR GROWTH. *Nature Reviews Cancer*. 2002;2(1):38–47. doi:10.1038/nrc704.
6. Bristow RG, Hill RP. Hypoxia and metabolism: Hypoxia, DNA repair and genetic instability. *Nature Reviews Cancer*. 2008;8(3):180–192. doi:10.1038/nrc2344.
7. Masson N, Ratcliffe PJ. Hypoxia signaling pathways in cancer metabolism: the importance of co-selecting interconnected physiological pathways. *Cancer Metab*. 2014;2(1):3. doi:10.1038/nature06734.
8. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nature Medicine*. 2004;10(8):789–799. doi:10.1038/nm1087.
9. Klinke DJ II. An evolutionary perspective on anti-tumor immunity. January 2013:1–13. doi:10.3389/fonc.2012.00202/abstract.
10. Emilsson V, Thorleifsson G, Zhang B, et al. Genetics of gene expression and its effect on disease. *Nature*. 2008;452(7186):423–428. doi:10.1038/nature06758.
11. Godoy-Ortiz A, Sanchez-Muñoz A, Chica Parrado MR, Álvarez M, Ribelles N, Dominguez AR, and Alba E. Deciphering HER2 Breast Cancer Disease: Biological and Clinical Implications. Front Oncol. 2019; 9: 1124. Published online 2019 Oct 29. doi: 10.3389/fonc.2019.01124
12. Bristow RG, Hill RP. Hypoxia and metabolism: Hypoxia, DNA repair and genetic instability. *Nature Reviews Cancer*. 2008;8(3):180–192. doi:10.1038/nrc2344.
13. Pouysségur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature Cell Biology*. 2006;441(7092):437–443. doi:10.1038/nature04871.
14. Emily F, Christoph L, Schofield CJ. *Hypoxic Response and Associated Diseases*. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2007. doi:10.1002/9780470048672.wecb232.
15. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell*. 2011;144(5):646–674. doi:10.1016/j.cell.2011.02.013.
16. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database Hallmark Gene Set Collection. *Cell Systems*. 2015;1(6):417–425. doi:10.1016/j.cels.2015.12.004.
17. Morris DS, Tomlins SA, Rhodes DR, Mehra R, Shah RB, Chinnaiyan AM. Integrating biomedical knowledge to model pathways of prostate cancer progression. *Cell Cycle*. 2007;6(10):1177–1187. doi:10.4161/cc.6.10.4247.
18. Clarke C, Madden SF, Doolan P, et al. Correlating transcriptional networks to breast cancer survival: a large-scale coexpression analysis. *Carcinogenesis*. 2013;34(10):2300–2308. doi:10.1093/carcin/bgt208.
19. Semenza, G. L. Targeting HIF-1 for cancer therapy. *Nature Reviews Cancer*, 2003, 3(10), 721–732. http://doi.org/10.1002/cncr.10774
20. Zhong, H., De Marzo, A. M., Laughner, E., Lim, M., Hilton, D. A., Zagzag, D., et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Research*, 1999, 59 (22), 5830–5835.
21. Quackenbush J. Microarray data normalization and transformation. *Nature Genetics*. 2002;32(Supp):496–501. doi:10.1038/ng1032.
22. Simon R. Microarray-based expression profiling and informatics. *Current Opinion in Biotechnology*. 2008;19(1):26–29. doi:10.1016/j.copbio.2007.10.008.
23. Ideker T, Thorsson V, Siegel AF, Hood LE. Testing for differentially-expressed genes by maximum-likelihood analysis of microarray data. *Journal of Computational Biology*. 2000;7(6):805–817. doi:10.1089/10665270050514945.
24. Reimers M. Making Informed Choices about Microarray Data Analysis. Lewitter F, ed. *PLoS Comput Biol*. 2010;6(5):e1000786. doi:10.1371/journal.pcbi.1000786.s001.
25. Chen K-H, Wang K-J, Tsai M-L, et al. Gene selection for cancer identification: a decision tree model empowered by particle swarm optimization algorithm. *BMC Bioinformatics*. 2014;15(1):1–10. doi:10.1186/1471-2105-15-49.
26. Bild AH, Parker JS, Gustafson AM, et al. An integration of complementary strategies for gene-expression analysis to reveal novel therapeutic opportunities for breast cancer. *Breast Cancer Res*. 2009;11(4):R55. doi:10.1186/bcr2344.
27. Salomonis N, Hanspers K, Zambon AC, et al. GenMAPP 2: new features and resources for pathway analysis. *BMC Bioinformatics*. 2007;8(1):217. doi:10.1186/1471-2105-8-217.
28. van't Veer LJ, Dai HY, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature*. 2002;415(6871):530–536.
29. Grisaru D, Hauspy J, Prasad M, et al. Microarray expression identification of differentially expressed genes in serous epithelial ovarian cancer compared with bulk normal ovarian tissue and ovarian surface scrapings. *Oncol Rep*. 2007;18(6):1347–1356.
30. Glinsky GV, Berezovska O, Glinskii AB. Microarray analysis identifies a death-from-cancer signature

predicting therapy failure in patients with multiple types of cancer. *J Clin Invest*. 2005;115(6):1503–1521. doi:10.1172/JCI23412DS1.

31. Lapointe J, Li C, Higgins JP, et al. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proc Natl Acad Sci USA*. 2004;101(3):811–816. doi:10.1073/pnas.0304146101.
32. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. 2005;102(43):15545.
33. Abatangelo L, Maglietta R, Distaso A, et al. Comparative study of gene set enrichment methods. *BMC Bioinformatics*. 2009;10(1):275. doi:10.1186/1471-2105-10-275.
34. Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Research*. 2011;40(D1):D109–D114. doi:10.1093/nar/gkr988.
35. Alexeyenko A, Sonnhammer ELL. Global networks of functional coupling in eukaryotes from comprehensive data integration. *Genome Research*. 2009;19(6):1107–1116. doi:10.1101/gr.087528.108.
36. Okawa S, Angarica VE, Lemischka I, Moore K, del Sol A. A differential network analysis approach for lineagespeci. *Nature Publishing Group*. November 2015:1–8. doi:10.1038/npjsba.2015.12.
37. Wang E, Zaman N, Mcgee S, Milanese J-S, Masoudi-Nejad A, O'Connor-McCourt M. Seminars in Cancer Biology. *Seminars in Cancer Biology*. 2015;30:4–12. doi:10.1016/j.semcancer.2014.04.002.
38. Restifo NP, Smyth MJ, Snyder A. Acquired resistance to immunotherapy and future challenges. *Nature Reviews Cancer*. 2016;16(2):121–126. doi:10.1038/nrc.2016.2.
39. Bui JD, Schreiber RD. Cancer immunosurveillance, immunoediting and inflammation: independent or interdependent processes? *Current Opinion in Immunology*. 2007;19(2):203–208. doi:10.1016/j.coi.2007.02.001.
40. McNutt M. Cancer Immunotherapy. *Science*. 2013;342(6165):1417–1417. doi:10.1126/science.1249481.
41. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic Cell Death in Cancer Therapy. *Annu Rev Immunol*. 2013;31(1):51–72. doi:10.1146/annurev-immunol-032712-100008.