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Identification of key genes and pathways involved in delayed wound healing among bleomycin using cancer patients via bioinformatics analysis

Author: Mutebi John Kenneth[1*]

Email: mutebi.john.kenneth@g.bracu.ac.bd

Affiliation; BRAC University, Dhaka-Bangladesh[1]

Department of Mathematics and Natural Sciences, Biotechnology Program

Dhaka, Bangladesh –Postal Code; 1212, Mohakhali –Dhaka.

Corresponding Author: mutebi.john.kenneth@g.bracu.ac.bd[*]

Abstract

This study aims at identifying differentially expressed genes (DEGs) upon bleomycin treatment among cancer patients and investigating their potential pathways. Candidate genes were identified from microarray datasets GSE94522 and GSE111043 from the Gene Expression Database and then using the GEO2R tool to select DEGs between bleomycin treated and non-treated lung tissues. Enrichr and Search Tool for the Retrieval Interacting Genes (STRING) were used to analyze the Kyoto Encyclopedia of Gene and Genome pathways and gene ontology and protein protein interactions (PPIs) of these DEGs. A total of 107 DEGs were co-expressed in both datasets and these were predominantly down-regulated in cell proliferation, metabolism and angiogenesis. 8 genes (EREG, TNC, IGF1, THBS4, THBS2, SPP1, AREG, and CDKN1A) were found to be significantly down-regulated due to bleomycin in the PI3K/Akt signaling pathway. Results hint at the fact that intervening therapies to cater for the resulting wounds and skin injuries should be

Keywords: Delayed wound Healing, Bleomycin, Differential Gene Expression, GEO2R, 27 STRING, and Chemotherapy

INTRODUCTION

Cancer is among the main types of non-communicable diseases (NCD) which claim 41 million people each year. As a result, several research studies have been dedicated to finding and/or improving therapies to treat the disease or reduce the side effects caused by existing therapies. Radiotherapy, surgery, and chemotherapy are the most commonly applied types of cancer treatments nowadays, depending on the patient's situation.

Although these treatments are highly non-specific and with various side effects, physicians continue to prescribe them to cancer patients after judging that the benefits to the patient are greater than the risk of side effects (Dower et al., 2020; Ganz, n.d.). Bleomycin is among the most used anti-cancer chemotherapy drugs and like other chemotherapies, it has several serious side effects including wound healing complications. Studies have enormously shown that bleomycin impairs the wound healing process. The mode of action of the majority of chemotherapeutics is based on inhibition of cell metabolism, cell division, and angiogenesis hence blocking various pathways responsible for effective wound repair. The present study aims at identifying key differentially expressed genes (DEGs) upon bleomycin exposure and investigating pathways of such genes for their involvement in the wound healing process.

METHODS

Gene expression profiles of GSE94522 (Yokoyama et al., 2017) and GSE111043 in bleomycin treated and normal control lung tissue cells were obtained from NCBI-GEO (https://www.ncbi.nlm.nih.gov/pubmed), a free public database of microarray/gene expression profiles. In this study, ethical approval was deemed unnecessary because the two datasets analyzed were publicly available. The analyzed datasets (GSE94522 and GSE111043) were on microarray platforms GPL10787 (Agilent-028005 SurePrint G3 Mouse GE 8x60K) and GPL21810 (Agilent074809 SurePrint G3 Mouse GE v2 8x60K), which included 3 bleomycin treated and 3 normal controls samples, and 2 bleomycin-treated and 4 untreated lung tissue samples respectively.

The DEGs between the bleomycin treated and normal control lung tissue cells were obtained after analyzing the two datasets with GEO2R, an online tool (http://www.ncbi.nlm.nih.gov/geo/geo2r/). For any gene to qualify as part of the DEGs, the log fold-change (FC) is greater than 2 and adjusted *P*-value < 0.05. Qualified genes from both datasets were then checked using an online tool Venn software to detect common DEGs between them. For down-regulated genes, log FC<–2 while log FC>2 represented up-regulated genes (Sean et al., 2007).

To identify unique biological properties of the DEGs, gene ontology analysis (GO) was performed, relating the results to an online collection of databases of genomes, diseases, biological pathways, drugs, and chemical materials called Kyoto Encyclopedia of Genes and Genomes (KEGG). The enrichment of DEGs in terms of biological process (BP), molecular function (MF), and cell component (CC) were visualized by the use of an online bioinformatics tool Database for Annotation, Visualization, and Integrated Discovery (DAVID). DEGs with P-value <0.05 were considered significant (Huang et al., 2009).

To identify interconnections between these DEGs, Protein-Protein Interaction (PPI) analysis was performed using the online PPI information evaluation tool Search Tool for the Retrieval of Interacting

Genes (STRING: https://string-db.org/) (Szklarczyk et al., 2014). Pathways to which DEGs belong were identified by analyzing the KEGG results and it was performed using Enrichr (http://amp.pharm.mssm.edu/Enrichr/). Enrichment analysis of these pathways was based on a cutoff value of $P<0.05$.

RESULTS

Identification of DEGs upon bleomycin treatment

The gene expression profiles of GSE94522 and GSE111043 were analyzed independently using GEO2R web-server analysis to identify DEGs. Two groups were defined for each dataset i.e.

"Bleomycin" treated and "Untreated". Basing on the pre-determined significant *P*-values and logFC values, a total of 341 and 595 DEGs respectively were identified. Further analysis with Venn diagram software revealed that 28 DEGs were co-expressed in both datasets and these were all downregulated genes i.e. logFC<–2.

Figure 1

***Figure 1**: DEGs from both datasets (GSE94522 and GSE111043) i.e. **-2 < LogFC > 2**. The 28 down-regulated DEGs were identified through Venna diagram software. (DEGs = differentially expressed genes)*

DEGs gene ontology and KEGG pathway analysis upon bleomycin treatment

The gene ontology (GO) analysis of the 28 common DEGs was done using DAVID software. It revealed that for Biological Processes (BP), most DEGs were down-regulated in the cell cycle, cell adhesion, and positive regulation of cell proliferation, mitotic nuclear division, and tissue remodeling. For molecular function (MF), DEGs were mostly down-regulated in protein binding, microtubule binding, and cytokine activity; and for cell components (CC), DEGs were significantly down-regulated on extracellular region/space and proteinaceous extracellular matrix.

Figure 1

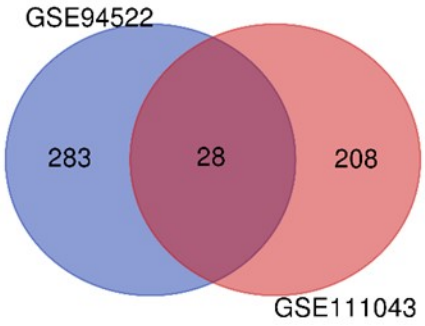

***Figure 1**: DEGs from both datasets (GSE94522 and GSE111043) i.e. **-2 < LogFC > 2**. The 28 down-regulated DEGs were identified through Venna diagram software. (DEGs = differentially expressed genes)*

Figure 2

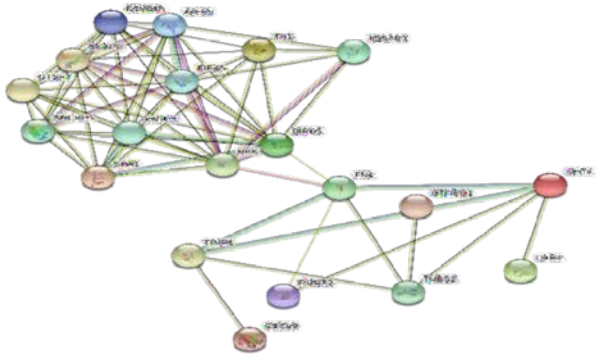

Figure 2: *Results of Protein-Protein-Interaction (PPI) analysis between common DEGs of both datasets. DEGs without interactions were eliminated from the figure.*

Analysis of KEGG revealed that DEGs were significantly down-regulated in the ECM-receptor interaction pathway, Focal adhesion pathway, and PI3K-Akt signaling pathway.

All the 28 DEGs were imported into STRING for which the parameters were set to multiple proteins and *Homo sapiens* as the preferred organism. The network stats indicated 26 nodes and 68 edges with an expected number of edges being 9. The results also showed co-expression of various DEGs basing on their RNA expression patterns, and protein co-regulation in humans and other organisms.

Table1: Gene Ontology for down-regulated DEGs

Category	Term	Count	Percentage	P.Value	FDR
GOTERM_CC_DIRECT	GO:0005578~proteinaceous extracellular matrix	5	20	3.85E-04	0.022736737
GOTERM_CC_DIRECT	GO:0005604~basement membrane	3	12	0.004813956	0.127296753
GOTERM_CC_DIRECT	GO:0005581~collagen trimer	3	12	0.006472716	0.127296753
GOTERM_CC_DIRECT	GO:0005819~spindle	3	12	0.010968502	0.161785399
GOTERM_CC_DIRECT	GO:0005576~extracellular region	7	28	0.015804326	0.186491048
GOTERM_CC_DIRECT	GO:0005615~extracellular space	6	24	0.02856822	0.280920829
GOTERM_CC_DIRECT	GO:0005876~spindle microtubule	2	8	0.05639942	0.413091469
GOTERM_CC_DIRECT	GO:0031012~extracellular matrix	3	12	0.057397483	0.413091469
GOTERM_CC_DIRECT	GO:0005881~cytoplasmic microtubule	2	8	0.065086131	0.413091469
GOTERM_CC_DIRECT	GO:0031093~platelet alpha granule lumen	2	8	0.070015503	0.413091469

Table2: **GO Biological process for down-regulated DEGs**

Category	Term	Count	Percentage	P.Value	FDR
GOTERM_BP_DIRECT	GO:0007067~mitotic nuclear division	6	24	2.28E-05	0.003574339
GOTERM_BP_DIRECT	GO:0007155~cell adhesion	7	28	3.57E-05	0.003574339
GOTERM_BP_DIRECT	GO:0051301~cell division	5	20	0.0014172	0.09448002
GOTERM_BP_DIRECT	GO:0022617~extracellular matrix disassembly	3	12	0.00523102	0.261551012
GOTERM_BP_DIRECT	GO:0007062~sister chromatid cohesion	3	12	0.009418041	0.376721625
GOTERM_BP_DIRECT	GO:0008284~positive regulation of cell proliferation	4	16	0.027888766	0.929625531
GOTERM_BP_DIRECT	GO:0045669~positive regulation of osteoblast differentiation	2	8	0.082376121	1
GOTERM_BP_DIRECT	GO:0016525~negative regulation of angiogenesis	2	8	0.085006749	1

Table3: **KEGG for Down-regulated DEGs**

Category	Term	Count	Percentage	P.Value	FDR
KEGG_PATHWAY	hsa04512:ECM-receptor interaction	5	20	1.09E-05	2.62E-04
KEGG_PATHWAY	hsa04510:Focal adhesion	5	20	3.20E-04	0.003840888
KEGG_PATHWAY	hsa04151:PI3K-Akt signaling pathway	5	20	0.00223218	0.017857442
KEGG_PATHWAY	hsa04145:Phagosome	3	12	0.027012473	0.162074837
KEGG_PATHWAY	hsa05144:Malaria	2	8	0.082270264	0.394897268

DISCUSSION

Cancer is a difficult disease to treat despite being so deadly. The available therapies today are surgery, and radiotherapy, chemotherapy, immunotherapy, hormone therapy, and stem cell therapy and are often used in combination with each other. However, chemotherapy is widely used not only in developing countries but also in the western world. The basic principle of chemotherapy can be thought of as a poison that kills cancer cells and healthy cells at a specific location in the body (Alfarouk et al., 2015). Bleomycin is one of such chemotherapeutic agents and it works by slowing or stopping the growth of cancer cells when a patient is injected with it into a vein, muscle, or under the skin or when placed in the space around the lungs through a chest tube by a health care professional (Šošić et al., 2020). Bleomycin side effects such as temporary hair loss, nausea, vomiting, weight loss, loss of appetite, chills, and fever are very important for patients and doctors when deciding the treatment plan (Müller et al., 2021). These aside, studies have enormously shown that bleomycin impairs the wound healing process. Basing on its mode of action which involves inhibition of cell metabolism, cell division, and angiogenesis, various pathways that are responsible for effective wound repair are blocked as a result (Deptuła et al., 2019). Therefore, identifying such pathways and finding suitable solutions can help to improve this cancer treatment strategy.

Two expression profile datasets GSE94522 and GSE111043 were analyzed using GEO2R and Venn software. A total of 28 common DEGs were found, basing on the pre-determined cut-offs logFC>2, logFC< -2, and adjusted P-value<.05. Gene ontology and pathway enrichment analysis which was done using DAVID and Enrichr methods showed that the 28 DEGs were particularly down-regulated in ECM-receptor interaction, Focal adhesion, and PI3K-Akt signaling pathways.
Studies have reportedly shown the involvement of these three pathways in the growth and progression of tumor cells (Bao et al., 2019; Ignjatović et al., 2021; Xu et al., 2020; Zhou et al., 2019). Focal-adhesion kinase (FAK) for instance mediates cell proliferation, growth-factor signaling, cell survival, and cell migration and these processes are characteristic features during malignancy development. In their study, (Lee et al., 2015; Yang et al., 2017) showed that FAK is involved in tumor formation and progression, while other studies have indicated an increased expression of FAK in human cancer. This means that FAK from the Focal-Adhesion pathway is a potential therapeutic target for any chemotherapy, including bleomycin (Dawson et al., 2021). The extracellular matrix serves as a scaffold upon which tissues are organized as well as being involved in other crucial biochemical and biomechanical capabilities that direct cell growth, survival, migration, and differentiation. These capabilities influence cancer hallmarks such as sustained proliferation, evasion of growth suppression, death resistance, replicative immortality among others hence making the ECM-receptor interaction pathway critical for malignancy and a target for any chemotherapy (Walker et al., 2018). The PI3K/AKT signaling pathway regulates cell survival and proliferation and its abnormal activation has been reported by different studies in various human cancers (Noorolyai et al., 2019), including breast, ovarian, prostate, and lung, making the pathway a vital target for bleomycin. Therefore, the down-regulation of genes involved in these pathways upon treatment with this common chemotherapeutic agent in cancer treatment is relatively understandable.

However, these pathways as well as DEGs that were found enriched in these pathways are very crucial in the wound healing process (Choi et al., 2015; Gao et al., 2016; Xue & Jackson, 2015). The process of wound healing is defined in three phases i.e. inflammation, proliferation, and remodeling (Tottoli et al., 2020). Upon any injury, blood clotting is immediately generated by clotinducing and regulating factors, allowing platelets to arrive at the injury site. These secrete more clotting factors and signal the

start of the inflammatory response to clear the wound site of foreign pathogens. This then enables the recruitment and proliferation of cells into granulation tissue hence filling the wound with newly synthesized cells (Nagai et al., 2018). As this tissue forms, angiogenesis starts to perfuse the area, eradicating the initial hypoxia state of the healing wound. The entire process involves cell adhesion, spreading, migration, proliferation, and apoptosis. Fibronectin (FN) encoded by the FN1 gene is actively involved in all stages but more significantly in the initial phase of wound healing during clot and extracellular matrix (ECM) formation. Other genes such as SPP1 (Kramerova et al., 2019), COL24A1, THBS2, and THBS4 were also found enriched in the ECM organization, a pathway that plays a central role by forming "scaffolding" which is an indispensable feature during tissue repair.

These genes were also found enriched in the PI3K/AKT signaling pathway. Studies have indicated that activation of the PI3K/AKT signaling pathway can be an alternative therapeutic approach for the treatment of skin injury. Inhibiting this pathway inhibits wound contraction, consequently delaying wound healing (Chen et al., 2020). Activation of this pathway allows migration of fibroblasts from normal tissue into the wound site, allowing key processes to take place for example breaking down the fibrin clot, creation of fresh extracellular matrix (ECM) and collagen structures to support those cells that are related to effective wound healing. Focal adhesion kinase (FAK) is a crucial tyrosine kinase non-receptor protein in cell proliferation and migration regulation during re-epithelialization process phase of wound healing (Dawson et al., 2021; Diaz Osterman et al., 2019; Lee et al., 2015). When its pathway is activated, cell migration and proliferation around the injured skin are accelerated hence accelerating the healing process. Although the ECM-receptor interaction pathway has been reported to play an important role in various cancer types, it is very crucial during cell proliferation, migration, differentiation, and survival in cutaneous wound healing (Szondi et al., 2021). ECM-receptor interactions indirectly influence the epidermal stem cell and more-differentiated reparative cell fate during reepithelialization through their role in modulating keratinocyte activity. ECM-receptor interaction signaling pathway also regulates growth factor bioavailability and activity. This is done by promoting cell-ECM interactions, mediating tissue stiffness, and regulating profibrogenic growth factor TGFβ1 (Knudsen et al., 2018). Chronic and non-healing wounds reportedly show loss of TGFβ1 which indicates that deregulating ECM-receptor interaction pathway will alter the healing process through this growth factor.

Conclusion

In conclusion, the obtained results implicate the 6 down-regulated DEGs (SPP1, COL24A1, THBS2, THBS4, FN1, and CXCL9) to play important roles during the wound healing process through their enriched pathways. Since these pathways are known to promote tumor progression in various cancer types, it is inevitable to halt their activity during cancer treatment. However, these predictions need to be verified through a series of experiments in various cancer types in the future. Nevertheless, the study hints at the fact that supplementary remedies should always be included in cancer management whenever chemotherapies such as bleomycin are prescribed to patients to cater for pre-existing wounds and/or skin injuries, or those that could occur during treatment.

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