

Genetic Expression of High Nitric Oxide Adapted Human Lung Adenocarcinoma Cell Line towards Angiogenesis

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Abstract

A549-HNO cells which adapt to high nitric oxide (HNO) proliferate at a higher rate and are more resistant to radiation when compared to A549 parent cells. This is due to an altered expression in several genes where key pathways are affected such as the process of apoptosis, cell proliferation and DNA transcription. A549 lung cancer cells that adapt to high concentrations of nitric oxide (NO) cause new mutations to occur by altering the cell's genome towards the angiogenesis pathway. The purpose of this study is to see the effect of HNO mutant cells and observe their growth potential behaviour in lung adenocarcinoma and their angiogenesis pathway. It was hypothesized that high NO will lead to more aggressive and resistant strains of mutant cells. Analysis has been conducted, using GO Biological Process (Uniprot) and 6.2% of the Gene Ontology enrichment analysis revealed that the angiogenesis signalling pathway was the most significantly enriched term within the Differentially Expressed Genes (DEGs). High NO mutant cells show aggressive angiogenic behaviour in tumor growth leading to more radio-resistant tumor cells in lung adenocarcinoma. In addition, these DEGs, can be potential targets for malignancies with aggressive growth and metastatic phenotype and clinical trials.

Keywords: Nitric Oxide (NO); High Nitric Oxide (HNO); Apoptosis; Angiogenesis; Adenocarcinoma; DEG (Differentially Expressed Genes)

Introduction

We have recently shown that high nitric oxide (HNO) adapted A549-HNO cells proliferate at a higher rate and are more radiation resistant when compared to A549 parent cells. In previous reports from our laboratory we have focused on the role of Nitric Oxide in human tumors and related gene products [1-26]. This is due to altered expression of several genes involved in key pathways specifically in the apoptotic process, cell proliferation and DNA transcription. Different patient's express different levels of NO within their tumor cells; the lower the NO expression, the more likely it is that chemotherapy/ radiation treatment will be successful whereas in high expressing tumor response and prognosis is poor. Knowing this, the parent A549 cell line and the adapted A549-HNO cell lines serve as a comparative model to study to effects of NO in tumor biology. The analysis is aimed to explore gene expression profile of differentially expressed genes to investigate the underlying mechanisms, and to identify novel targets. In this study, we hypothesize that the adaptation of A549 lung cancer cells to high concentrations of nitric oxide (NO) causes new mutations in the adapted cells' genome towards the angiogenesis pathway. As a malignant growth exceeds the size of a few 100 mm, nutrient diffusion becomes a growth-limiting factor. Hypoxia and cancer-specific genetic abnormalities, mainly by up-regulation of the hypoxia inducible factors, drive the secretion of proangiogenic factors and the suppression of antiangiogenic factors, allowing the tumor microenvironment favourable for angiogenesis to occur. This causes the formation of new blood vessels, and the existing blood vessels to be modified to provide blood supply to the tumor. The sprouting of blood

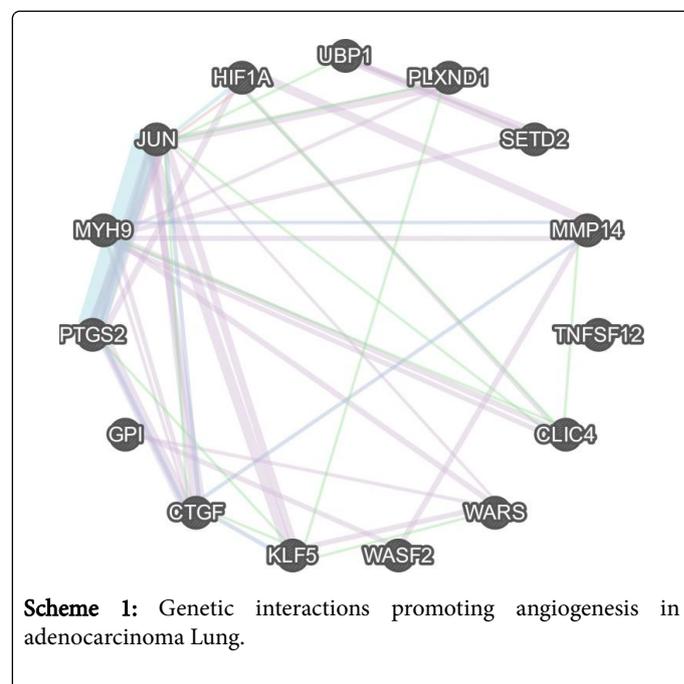
vessels from existing blood vessels is called angiogenesis [27,28]. Angiogenesis is one of the poor prognostic factors in cancer development. Diagnostic and treatment modalities are being studied to pick angiogenic genes and gene products in early stages and targeted treatments against those factors are being tested [28]. A549 cell lines were studied; these cell lines were adapted to high levels of NO by using the NO donor DETA-NONOate. Genomic DNA was isolated from A549-HNO and A549 parent cells using standard protocol. DNA microarrays were used to compare gene expression between the A549-HNO genome versus A549 parent cells genome. Method Parameters of FDR threshold p-value of <0.05 and fold change cut off of >2 for up regulation and -2 for down regulation. 535 genes were up regulated whereas 4745 were down regulated. Functional Enrichment Analysis has been conducted, using GO Biological Process (Uniprot) and 6.2% of the Gene Ontology enrichment analysis revealed that the angiogenesis signalling pathway was the most significantly enriched term within the DEGs. Finally, genetic interaction (GI) networks were constructed using Metscape and Cytoscape, to identify hub genes of the following genes in the Table 1A.

Gene	Full Name	Brief Description
CLIC4 (Figure 1)	Chloride intracellular channel 4 gene.	Protein coding gene involving in maintaining intracellular pH and regulation of cell volume.
CTGF (Figure 2)	Connective tissue growth factor gene.	Protein coding gene plays a role in chondrocyte proliferation and differentiation, cell adhesion in many cell types, and is related to platelet-derived growth factor.

GPI (Figure 3)	Glucose-6-phosphate isomerase gene.	The encoded protein has been identified as a moonlighting protein based on its ability to perform mechanistically distinct functions. In the cytoplasm, the gene product functions as a glycolytic enzyme (glucose-6-phosphate isomerase) that interconverts glucose-6-phosphate and fructose-6-phosphate. Extracellularly, the encoded protein (also referred to as neuroleukin) functions as a neurotrophic factor that promotes survival of skeletal motor neurons and sensory neurons, and as a lymphokine that induces immunoglobulin secretion. The encoded protein is also referred to as autocrine motility factor based on an additional function as a tumor-secreted cytokine and angiogenic factor.
HIF1A (Figure 4)	Hypoxia-inducible factor 1 alpha gene.	Plays an essential role in embryonic vascularization, tumor angiogenesis and pathophysiology of ischemic disease.
JUN (Figure 5)	Transcription factor AP-1 (Activator protein 1) gene	Encodes a protein which is highly similar to the viral protein, and which interacts directly with specific target DNA sequences to regulate gene expression.
KLF5 (Figure 6)	Kruppel-like factor 5 gene.	The encoded protein is a transcriptional activator that binds directly to a specific recognition motif in the promoters of target genes. This protein acts downstream of multiple different signaling pathways and is regulated by post-translational modification. It may participate in both promoting and suppressing cell proliferation.
MMPI4 (Figure 7)	Matrix metalloproteinase-14 gene.	Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis.
PLXND1 (Figure 8)	Plexin-D1 Precursor gene.	Protein coding gene.
PTGS2 (Figure 9)	Prostaglandin G/H synthase 2 Precursor gene.	This gene encodes the inducible isozyme. It is regulated by specific stimulatory events, suggesting that it is responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis.
SETD2 (Figure 10)	Histone-lysine N-methyltransferase gene.	This gene encodes a protein belonging to a class of huntingtin interacting proteins. Protein coding gene.
UBP1 (Figure 11)	Upstream-binding protein 1 gene.	Protein coding gene.

MYH9 (Figure 12)	Myosin-9 (Myosin heavy chain 9) gene.	The encoded protein is a myosin IIA heavy chain that contains an IQ domain and a myosin head-like domain which is involved in several important functions, including cytokinesis, cell motility and maintenance of cell shape.
WARS (Figure 13)	Tryptophanyl-tRNA synthetase, mitochondrial Precursor gene.	This gene encodes the mitochondrial tryptophanyl-tRNA synthetase.
WASF2 (Figure 14)	Wiskott-Aldrich syndrome protein family member 2 gene.	The gene product is a protein that forms a multiprotein complex that links receptor kinases and actin. Binding to actin occurs through a C-terminal verprolin homology domain in all family members. The multiprotein complex serves to transduce signals that involve changes in cell shape, motility or function.
TNFSF12 (Figure 15)	Tumor necrosis factor ligand superfamily member 12 gene.	This protein and its receptor are both found to be important for B cell development. <i>In vitro</i> experiments suggested that this protein may be able to induce apoptosis.

Table 1A: Primary genes responsible for angiogenesis in tumor microenvironment.



Scheme 1: Genetic interactions promoting angiogenesis in adenocarcinoma Lung.

Expression variance of different genes

The reason for the parent cell lines were adapted to HNO during the experimentation were to demonstrate the overall effects of the genes on angiogenesis as a whole and not individually. Studies have already established the individuality of genes and their functions causing angiogenesis. Regardless of the cancer cell lines used the outcome and

functionality doesn't change. Our goal was to determine how the 15 genes work together and their possible correlation. Ultimately their common effect and end result was angiogenesis.

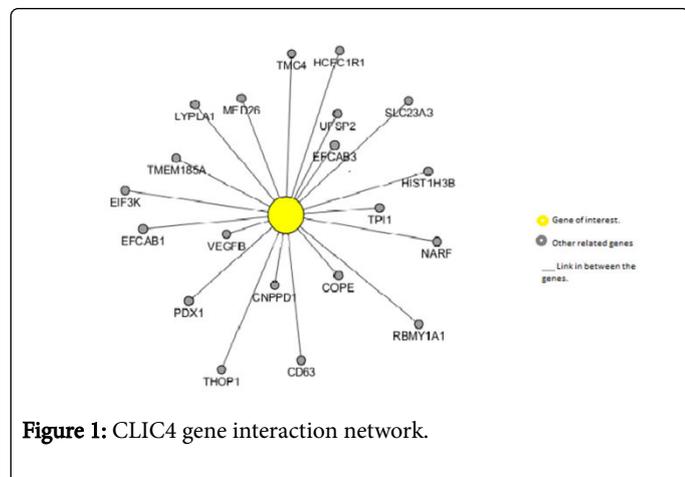


Figure 1: CLIC4 gene interaction network.

CLIC4: Plays a critical role in angiogenesis by supporting acidification of vacuoles along the cell-hollowing tubulogenic pathway [29]. New capillaries are formed through angiogenesis and an integral step in this process is endothelial tubulogenesis. The molecular mechanisms driving tube formation during angiogenesis are not yet delineated. Recently, the chloride intracellular channel 4 (CLIC4)-orthologue EXC-4 was found to be necessary for proper development and maintenance of the *Caenorhabditis elegans* excretory canal, implicating CLIC4 as a regulator of tubulogenesis. Reduced CLIC4 expression decreased cell proliferation, capillary network formation, capillary-like sprouting, and lumen formation. This suggests that normal endogenous CLIC4 expression is required for angiogenesis and tubulogenesis. Accordingly, increased CLIC4 expression promoted proliferation, network formation, capillary-like sprouting, and lumen formation. We conclude that CLIC4 functions to promote endothelial cell proliferation and to regulate endothelial morphogenesis, and is thus involved in multiple steps of *in vitro* angiogenesis [29-35].

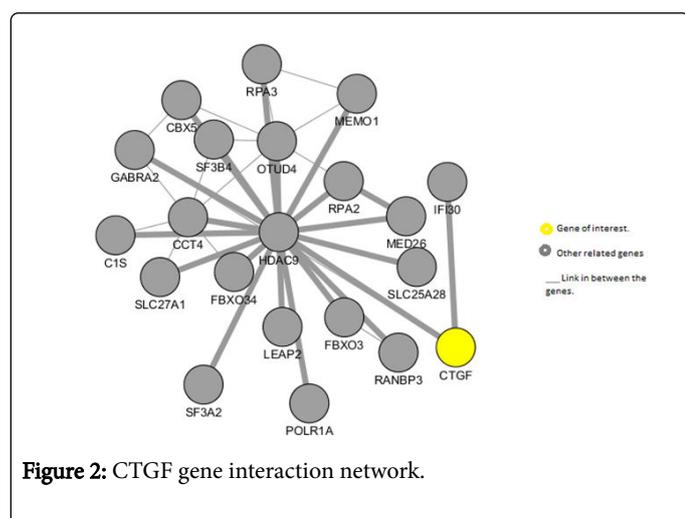


Figure 2: CTGF gene interaction network.

CTGF: VEGF-A utilizes the connective tissue growth factor (CTGF)/formyl peptide receptor-like 1 (FPRL1) axis as one of its mediators in angiogenesis [36]. Connective tissue growth factor (CTGF) is an epigenetically regulated growth factor with functions in

angiogenesis and cell-matrix interactions and plays a pivotal role in hepatocellular carcinoma (HCC) [37,38]. CTGF activates PI3K, AKT, ERK, and NF- κ B/ELK1 pathway, leading to the upregulation of miR-210, contributing to inhibit GPD1L expression and prolyl hydroxylases 2 activity, promoting HIF-1 α -dependent VEGF expression and angiogenesis in human synovial fibroblasts [39].

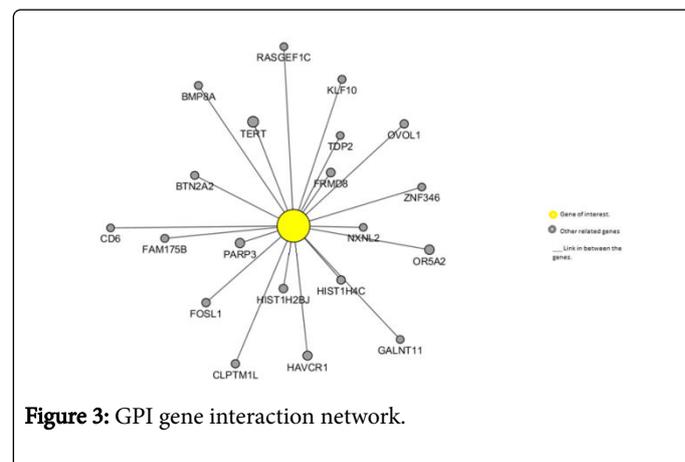


Figure 3: GPI gene interaction network.

GPI: T-cadherin (T-cad) is an atypical GPI-anchored member of the cadherin superfamily. In vascular tissue, T-cad expression is increased during atherosclerosis, restenosis, and tumor neovascularization. *In vitro*, overexpression and/or haemophilic ligation of T-cad on endothelial cells (ECs) facilitate migration, proliferation, and survival [40] although certain recent studies have shown results contrary to this. Recently it was shown that Beta-2 glycoprotein I (beta 2 GP1) is a plasma glycoprotein which interacts with various proteins of the coagulation and fibrinolysis system. Beta 2 GP1 has recently been shown to have anti-angiogenic properties [41,42].

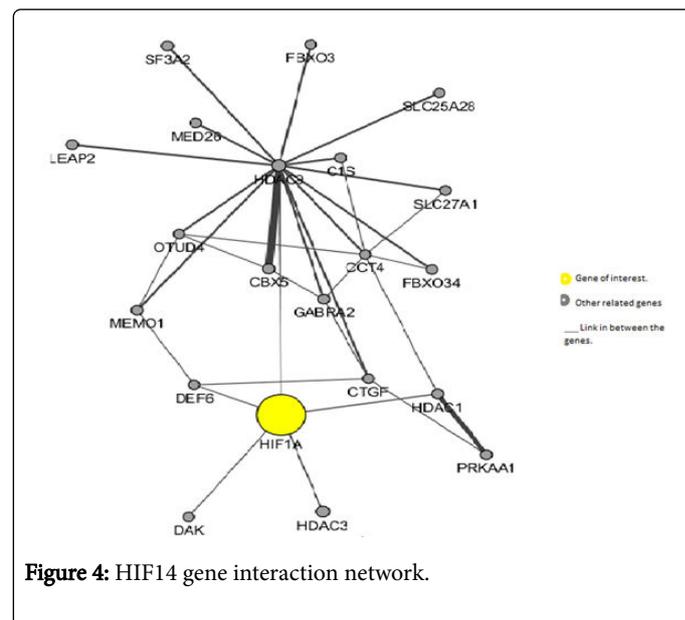
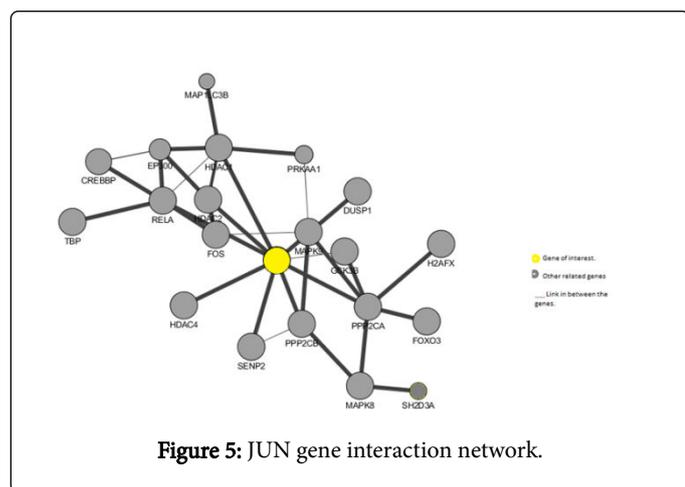


Figure 4: HIF14 gene interaction network.

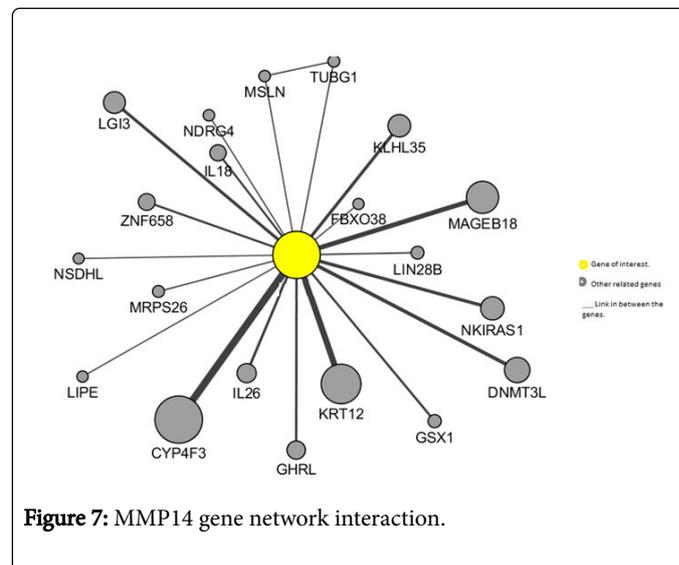
HIF1A: The hypoxia-inducible transcription factor, HIF-1, adapts cells to low oxygen tension. In addition to the activation of angiogenesis by induction of VEGF, HIF-1 may trigger hypoxia-induced growth arrest and apoptosis. Tumors derived from HIF-1-alpha-overexpressing cells revealed an increase in apoptosis when

compared to control tumors, despite a marked increase in vascular density. We conclude that in lung A549 cells, overexpression of HIF-1alpha negatively affects tumor growth due to decreased proliferation and increased apoptosis, despite augmented angiogenesis [32,33]. The formation of new blood vessels is regulated by the hypoxia-inducible factor 1 (HIF-1). The expression of HIF-1 correlates with hypoxia-induced angiogenesis as a result of the induction of vascular endothelial cell growth factor (VEGF) [34,35].

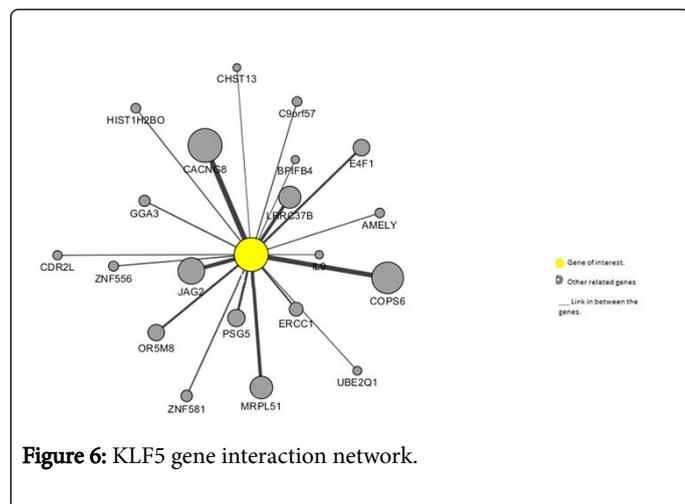
KLF5 plays in regulation of CSCs in HCC remains to be elucidated [46]. Krüppel-like factors (KLF) are a family of transcriptional regulators. They are involved in diverse cellular processes, such as proliferation, apoptosis, and angiogenesis among others [47].



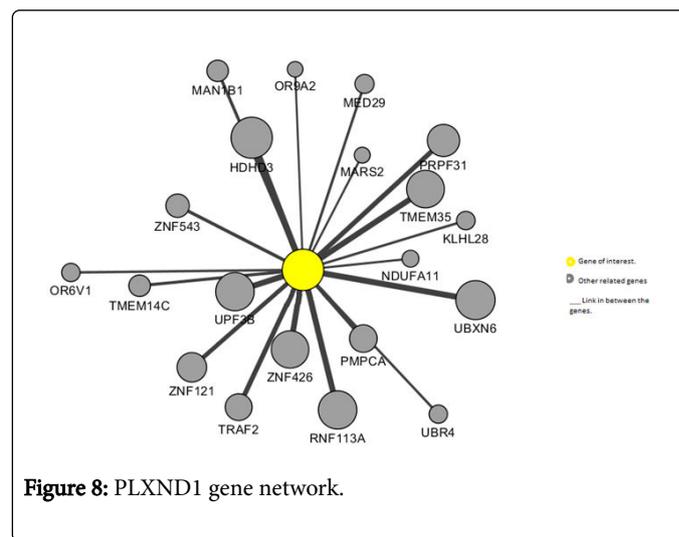
JUN: c-Jun is a component of the transcription factor activator protein 1 (AP-1), which binds and activates transcription at TRE/AP-1 elements. Extra- or intracellular signals, including growth factors, transforming oncoproteins, and UV irradiation, stimulate phosphorylation of c-Jun at serine 63/73 and activate c-Jun-dependent transcription. Therefore, activated c-Jun potentially plays an important role in carcinogenesis and cancer progression [43] activated c-Jun is predominantly expressed at the invasive front in breast cancer as well as urothelial cancer [44] and is associated with proliferation and angiogenesis. Earlier studies have established a functional, *in vitro* link between activated c-Jun and tumor angiogenesis. (Figures 1-11).



MMP14: Matrix metalloproteinase 14 (MMP-14) is the only membrane-anchored MMP that plays critical roles in tumorigenesis and aggressiveness [48]. Hepatocyte nuclear factor 4 alpha (HNF4a) exhibits oncogenic activity that affects the aggressiveness and angiogenesis of Neuroblastoma through activating the transcription of MMP-14. Also, elevated matrix metalloproteinase-14 expression correlates with invasive characteristics of invasive pituitary adenoma [49]. Even surface expression of matrix metalloprotease (MMP)-14 on ovarian cancer cells stimulates a tumor-stromal signalling pathway that promotes angiogenesis and tumor growth [50].



KLF5: KLF5 promotes angiogenesis of bladder cancer through directly regulating VEGFA transcription [45]. Krüppel-like factor 5 (KLF5) regulates many factors involved in cell cycle, migration, inflammation, angiogenesis and stemness and has cancer-promoting effects in some cancers. While some reports have indicated that KLF5 may have important roles in regulation of CSCs, what role, if any,



PLXND1: Blood vessel networks are typically formed by angiogenesis, a process in which new vessels form by sprouting of endothelial cells from pre-existing vessels. This process is initiated by vascular endothelial growth factor (VEGF)-mediated tip cell selection and subsequent angiogenic sprouting. Surprisingly, we found that VEGF directly controls the expression of Plexin-D1, the receptor for

the traditional repulsive axon guidance cue, semaphorin 3E (Sema3E) [51].

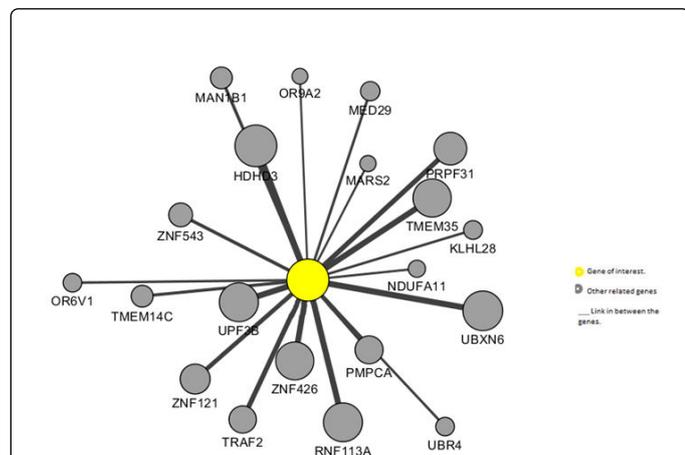


Figure 9: PTGS2 gene interaction network.

PTGS2: Is associated with increased cell adhesion, phenotypic changes, resistance to apoptosis and tumor angiogenesis. In cancer cells, PTGS2 is a key step in the production of prostaglandin E2 (PGE2), which plays important roles in modulating motility, proliferation and resistance to apoptosis [52]. There is strong evidence that PTGS2 is expressed in most solid tumor types. Cellular expression of PTGS2 is increased in the earliest stages of carcinogenesis and through tumor development and invasive tumor growth. A large number of studies have examined that PTGS2 up regulation is particularly common and associated with worse survival among colon cancer patients. PTGS2 genotype is also be found associated with breast cancer, biliary tract cancer and colorectal cancer [52]. PTGS2 CpG island hypermethylation portended an increased risk of recurrence in human prostate cancer [52]. In PTGS2 positive pancreatic cancer, the nimesulide-induced increase of VEGF production by the cancer cells was offset by a decrease in VEGF production by the non-malignant cell types leading to reduced tumor angiogenesis and growth [52]. PTGS2 overexpression is observed in breast cancer and has been associated with indicators of poor prognosis, such as lymph node metastasis, poor differentiation, and large tumor size [52].

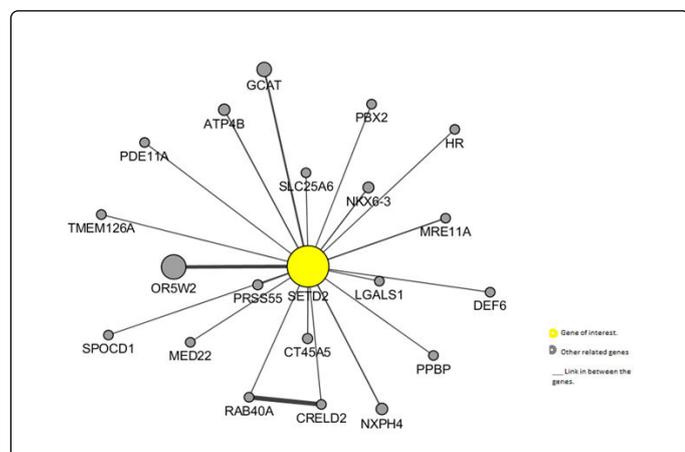


Figure 10: SETD2 gene interaction network.

SETD2: HYPB is a human histone H3 lysine 36 (H3K36)-specific methyltransferase [53,54]. There are only handful of studies done to find out the mechanism and role of SETD2 as a contributor of angiogenesis.

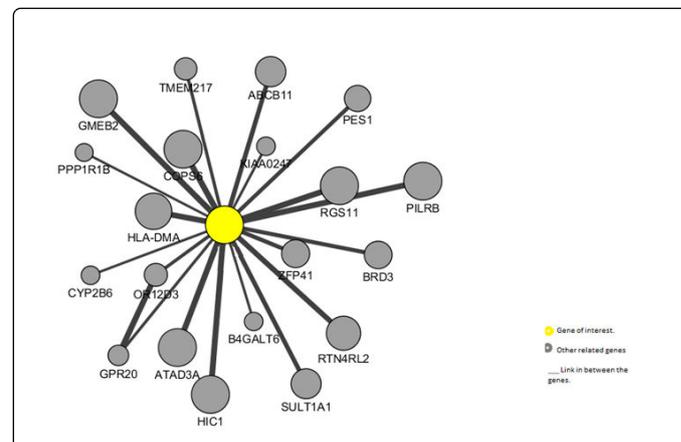


Figure 11: UBP1 gene interaction network.

UBP1: (Ustilago binding protein one), binds preferentially to DNA molecules lacking chain interruptions. The introduction of DNA breaks by a restriction enzyme or a purified nuclease, from Ustilago maydis, causes the dissociation of protein-DNA complexes. UBP1 stimulates the relaxation of negatively supercoiled DNA, mediated by Ustilago type I topoisomerase, through a mechanism most likely involving the association of UBP1 with the DNA rather than with the topoisomerase. The prebinding of UBP1 to DNA templates, subsequently assembled into minichromosomes, results in the development of a disorganized nucleosomal array. Possible roles for UBP1 in processes that involve changes in DNA topology, such as chromatin assembly, could lead to the angiogenesis in the tumor microenvironment [55,56].

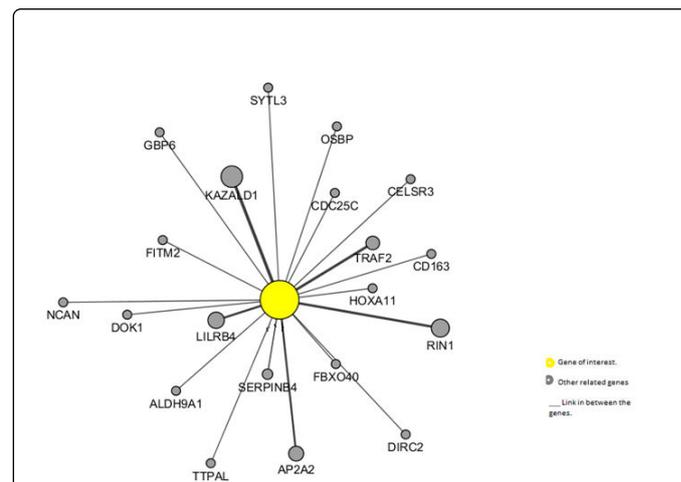


Figure 12: MYH9 gene interaction network.

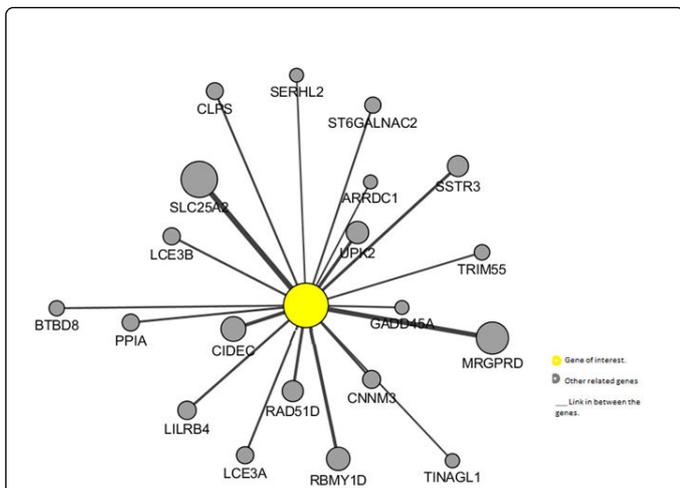


Figure 13: WARS gene interaction network.

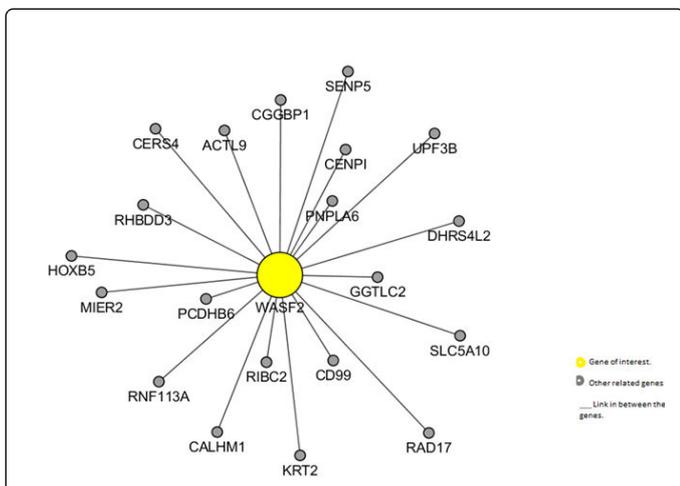


Figure 14: WASF2 gene interaction network.

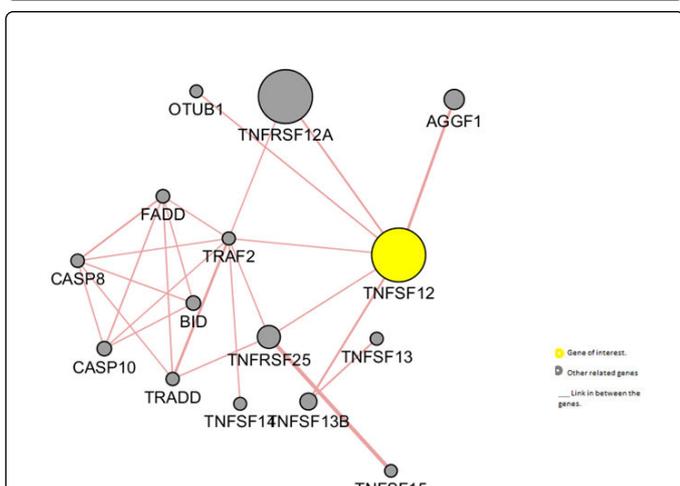


Figure 15: TNFSF12 gene interaction network.

Figures 12-15 are genes which were MYH9, WARS, WASF and TNFSF12. The aforementioned genes haven't had enough studies done but these genes contribute to angiogenesis along with other key genes and they should be further studied.

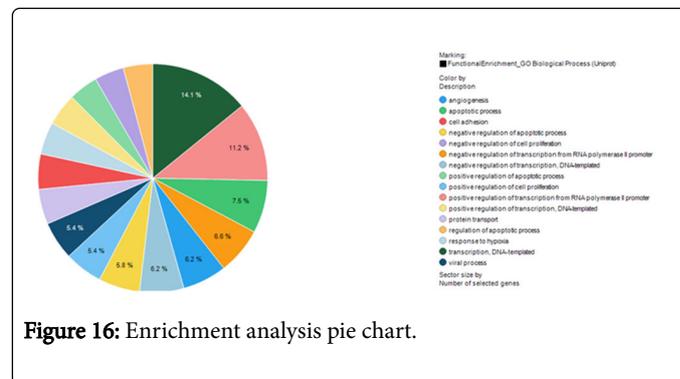


Figure 16: Enrichment analysis pie chart.

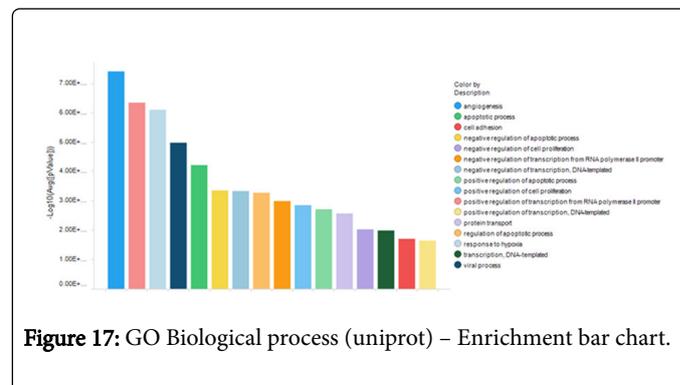


Figure 17: GO Biological process (uniprot) - Enrichment bar chart.

Figures 16 and 17, Enrichment bar chart analysis identified 16 significant pathways; majority can be divided into the regulation of apoptotic process, cell proliferation and DNA transcription. Angiogenesis is the most significant out of all the processes and figure 18 shows the fold change expression of the significant genes. In addition, these DEGs, can be potential targets for malignancies with aggressive growth and metastatic phenotype.

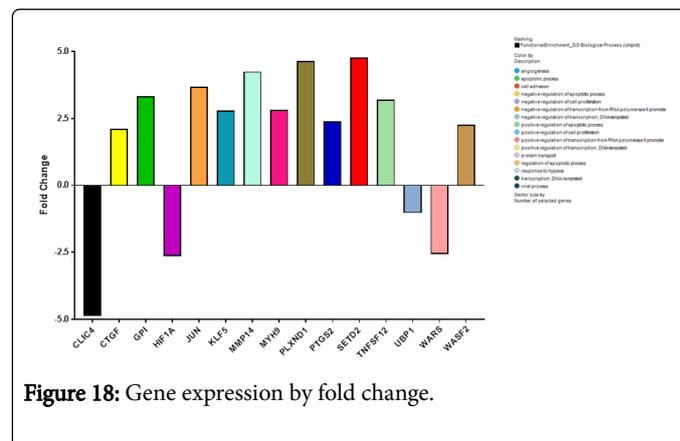


Figure 18: Gene expression by fold change.

Discussion

The main focus of our study was to determine various genes and their expressions for future targeted-therapy. Nitric Oxide expression by tumor cells is indirectly proportional to the response on

chemotherapy and radiation. The advanced techniques and parameters used to gather the information and compare them during the experimentation helped established accurate data. Furthermore, GO Biological Process (Uniprot) helped aid in deducing angiogenesis as the most functionally expressed biological process. The A549-HNO cells show enhanced growth behaviour in harsh conditions. Also, as presented by gene interaction networking analysis of the genomes of HNO and Parent cell lines it showed angiogenesis regulatory genes to be upregulated. A variety of genes were tested such as the underexpression of CLIC4 affecting angiogenesis by tubule formation and endothelial proliferation. Another involved gene was the overexpression of CTGF which played an important role in hepatocellular carcinoma by activating a series of genes causing angiogenesis. The GP1 gene in vascular tissue was overexpressed during tumor neovascularization assisting in migration and proliferation. The HIF1A gene was under-expressed in lung cancer cells causing hypoxia-induced angiogenesis with the help of VEGF. An activated JUN gene in breast and urothelial cancer had been linked to tumor angiogenesis and proliferation as well. While the overexpression of KLF5 activates many cancer-promoting factors it was the direct effect on VEGFA that led to the angiogenesis seen in bladder cancer. The MMP14 gene when overexpressed demonstrated various oncogenic effects in neuroblastoma cells and ovarian cancer cells via a tumor-stromal signalling pathway. When the PLXND1 gene was overexpressed it promoted angiogenesis by angiogenic sprouting facilitated by VEGF. The overexpression of PTGS2 had multiple cancer-promoting factors which can be attributed to the production of PGE2. This gene had shown multiple effects in breast, biliary, colorectal, and pancreatic cancer. Overexpression of the STED2 gene has shown contribution towards angiogenesis but the extent isn't well known yet due to the scarce amount of studies done. The under expression of the UBP1 gene may affect the unwinding of supercoiled DNA and in chromatin assembly leading to angiogenesis. As for the WARS and WASF genes not enough studies have been conducted to conclude the specifics but these genes play roles in angiogenesis and require future studies to be performed and are potential target for clinical trials. This study showed strong association of certain genes playing pivotal role in angiogenesis in Adenocarcinoma Lung although this association requires validation in future studies.

Acknowledgments

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