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GENE EXPRESSION PROFILE OF APATINIB RESISTANCE IN NON-SMALL CELL LUNG CANCER: INTEGRATIVE BIOINFORMATICS FOR THERAPEUTIC INSIGHTS

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ABSTRACT

Non-Small Cell Lung Cancer (NSCLC) is the predominant form of lung cancer, yet the development of drug resistance poses substantial challenges to its treatment, particularly with the use of Apatinib, a tyrosine kinase inhibitor that offer targeted therapies. This study aims to explore the molecular mechanisms underlying Apatinib resistance in NSCLC through integrative bioinformatics analyses of the publicly available transcriptomics data from the NCBI Gene Expression Omnibus (GEO) database. Specifically, tools GEO2R and Gene Set Enrichment Analysis (GSEA) were applied to identify differentially expressed genes and significant pathways associated with drug resistance by comparing the transcriptomics data across NSCLC lines: the sensitive PC-9 cells, the Gefitinib-resistant PC-9 (PC9GR) cells, and their respective Apatinib-treated counterparts. Key findings included but were not limited to that genes such as ARHGAP28 and TLR4 were significantly upregulated in Gefitinib-resistant cells, while SALL2 and MAP9 were downregulated. The critical pathways in resistance might involve fatty acid metabolism, bile acid metabolism, and Notch signaling. The intersection of differentially expressed genes across various comparisons has highlighted potential biomarkers and therapeutic targets to overcome Apatinib resistance. This study provides comprehensive insights into the gene expression changes and pathway alterations associated with Apatinib resistance in NSCLC. Further investigations, including in vivo studies, are essential to validate these potential targets and improve treatment efficacy.

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INTRODUCTION

Non-small cell Lung Cancer (NSCLC) is the most common type of lung cancer, accounting for approximately 85% of all cases (Brahmer *et al*., 2018). Despite advances in targeted therapies, the development of drug resistance remains a significant obstacle to effective treatment (Zhang *et al*., 2024). One such targeted therapy is Apatinib, a tyrosine kinase inhibitor that has shown promise in treating various cancers, including NSCLC (Chen *et al*., 2023). Apatinib works by inhibiting the vascular endothelial growth factor receptor-2 (VEGFR-2), thereby hindering tumor angiogenesis and growth. However, the efficacy of Apatinib is often hampered by the emergence of resistance mechanisms within cancer cells (Li *et al*., 2022), necessitating further investigation into the resistance pathways. Earlier studies have endeavored to examine the molecular mechanisms of drug resistance in NSCLC, particularly regarding tyrosine kinase inhibitors like Gefitinib and Apatinib (Sierra *et al*., 2010). Moreover, genes such as ARHGAP28 and CYP26A1 have been implicated with a link to drug resistance (Osanai & Lee, 2014; Yeung *et al*., 2014). This study will further explore the potential genes involving in resistance to Apatinib. Several studies have used GEO2R and GSEA analysis, finding meaningful differential gene expression and pathway enrichment related to Gefitinib resistance with subsequent treatment of Apatinib in NSCLC cell lines (Wei *et al*., 2020; Wu & Xi, 2021). However, there remain less explored pathways, which are studied as potential therapeutic targets in this study. Although NSCLC diagnosis and treatment have evolved, drug resistance remains to be one major obstacle to the effectiveness of Apatinib treatment after long-term use. Previous studies have identified various molecular pathways and genetic mutations related to such resistance (Meador & Hata, 2020; Xue *et al*., 2017). However, gaps remain in fully understanding the roles of fatty acid metabolism, bile acid metabolism, and Notch signaling in Apatinib resistance.This study aims to bridge these gaps through a dual approach by utilization of integrative bioinformatics tools including GEO2R, R Studios, and gene set enrichment analysis (GSEA). It improves the understanding of these resistance mechanisms. It opens new avenues for designing a range of directed therapies to circumvent this resistance, ultimately improving the clinical management of NSCLC patients.

MATERIALS & METHODS

Gene Dataset Associated with Cell Lines: Publicly available transcriptomics data related to the drug resistance of Gefitinib and Apatinib in NSCLC were retrieved from the NCBI Gene Expression Omnibus (GEO) database (Accession# GSE129221). This dataset was

generated on the Illumina platformconsisting of RNA-seq reads of sensitive PC-9 cell lines (PC9, n = 3), Gefitinib-resistant PC-9 cells (PC9GR, n = 3), sensitive PC-9 cells treated with Apatinib (PC9-Apa), and Gefitinib-resistant PC-9 cells treated with Apatinib (PC9GR-Apa, n = 3) (Song *et al*., 2019a).

Differential Gene Expression Analysis with GEO2R: GEO2R (www.ncbi.nlm.nih.gov/geo/geo2r/) is an interactive web-based tool provided by GEO. Itwas utilized to process and analyze the raw gene expression data. GEO2R performs differential gene expression analysis using the limma package, which accounts for experimental design and variability. The tool identifies genes differentially expressed across PC9 and PC9GR groups. Default parameters were used, and genes with an adjusted p-value (FDR-corrected) less than 0.05 were considered statistically significant. Specifically, log2 fold change value (LFC) more than 1.5 (less than -1.5) was detected as upregulated (downregulated).

Gene Set Enrichment Analysis: GSEA was conducted using the Broad Institute GSEA software (www.gsea-msigdb.org/gsea/). The analysis was run with 1,000 permutations, and gene sets with a false discovery rate (FDR) q-value less than 0.25 were considered significantly enriched. Hallmark and KEGG gene sets were used to identify significant pathways involved in drug resistance and response mechanisms.

Data Presentation: All data analysis and statistical computations were performed under R (version 4.1.0). UMAP plots were generated using the umap package (version 0.2.7.0). Bar plots summarizing GSEA results were generated using ggplot2 (version 3.3.5). Volcano plots were generated with the Enhanced Volcano package (version 1.10.0). The Venn diagram illustrating common and unique gene expression changes across different comparisons was generated using interactive (Heberle *et al*., 2015). R and packages above are integrated with GEO2R analysis tool.

RESULTS & DISCUSSION

Through the integration of GSEA and GEO2R analysis, this study investigated molecular changes in gene expression and concordant pathways associated with Non-Small Cell Lung Cancer PC9 sensitive and Gefitinib-resistant cell lines treated with Apatinib, and the results are outlined below for the key findings.

Comparison 1: PC9 vs. Gefitinib-Resistant PC9 (PC9GR): In comparing the parental PC9 cell line and the Gefitinib-resistant PC9 cell line (PC9GR), 2874 genes were found to be differentially expressed. ARHGAP28 and TLR4 were among the most upregulated genes in the resistant line. ARHGAP28 activates Rho GTPase, affecting cell morphology and motility, potentially contributing to changes in cell movement and structural integrity in resistant cells. TLR4 plays a critical role in the immune response by recognizing pathogens and activating inflammatory pathways, possibly influencing the tumor microenvironment and aiding immune evasion. The identification of these two genes confirms previous studies that TLR4 plays a role in drug resistance (Kashani, Zandi, Pourbagheri-Sigaroodi, Bashash, & Ghaffari, 2021). The most remarkably downregulated genes in the resistant line were SALL2 and MAP9. SALL2 is a transcription factor with numerous functions in development and tumor suppression; its downregulation could potentially reduce its tumor-suppressive activities, favoring resistance. MAP9 is a microtubule-associated protein relevant to cell division; its downregulation could disrupt normal cell division processes, thus favoring resistance mechanisms. Identification of these two genes downregulated in the resistant line confirms previous studies that both are related to drug resistance of cancer cells (Ye *et al*., 2019; Li, Wang, Gao, Zhang, & Zhang, 2024). Pathway enrichment analysis using Hallmark gene sets identified significant pathways in PC9GR cell lines, such as fatty acid metabolism, bile acid metabolism, Notch signaling, peroxisome, and complement system pathways.

Notch signaling, fatty acid metabolism, and bile acid metabolism pathways were upregulated. KEGG analysis identified pathways related to retinol metabolism, ABC transporters, and Ascorbate and Aldarate metabolism. Previous studies showed ABC transporters, Ascorbate and Aldarate pathways were related to drug resistance (Ahmed Laskar & Younus, 2019; Fletcher, Williams, Henderson, Norris, & Haber, 2016; Liu *et al*., 2019).

Comparison 2: PC9 vs. PC9 Treated with Apatinib: Comparing the parental cell line PC9 to the PC9 cell line treated with Apatinib revealed 402 genes significantly differentially expressed. Among the most upregulated genes in the cell line treated with Apatinibwere FAM30A and CYP26A1. FAM30A, although its exact functional role is not particularly clear, may have some role in regulating cell growth (Ye& Chen, 2024). CYP26A1 is a retinoic acid metabolizer that modulates cell differentiation and proliferation, possibly affecting the cellular response against Apatinib (Osanai, Sawada & Lee, 2010). Significantly upregulated pathways detected by GSEA in Apatinib-treated PC9 cells included MTORC1 signaling, TGF beta signaling, MYC targets v1 and Notch signaling. Conversely, protein secretion pathways, bile acid metabolism, and inflammatory response were downregulated in those treated cells.

Comparison 3: PC9GR vs. PC9GR Treated with Apatinib: A comparison between Gefitinib-resistant PC9 cells and the PC9GR treated with Apatinib found 1175 genes showing significantly differential expressions. The top upregulated genes in the Apatinib-treated line were DIO2-AS1 and SAMD15. The gene function of SAMD15 is not well characterized, but DIO2-AS1 is a noncoding RNA that may be involved in controlling thyroid hormone metabolism and, thus, cellular metabolic control (Yang, 2022). The most downregulated genes in the Apatinib-treated line were HSPA6 and CYP26A1. HSPA6 is a heat shock protein involved in protein folding and stress response; its downregulation could impact cellular stress responses (Shorbagi& Brown, 2016), with an additional impact on retinoic acid metabolism and cell differentiation. Pathway analysis demonstrated that Apatinib-treated PC9GR cells were highly enriched in cell cycle-linked pathways, including the G2M checkpoint, E2F targets, and mitotic spindle. Additionally, pathways of apoptosis were also significantly enriched. KEGG pathway analysis indicated differences in cell cycle and DNA replication pathways between cells.

Comparison 4: Treatment of Apatinib on PC9 and PC9GR: The comparison between the treatment on Apatinib on PC9 and PC9GR cells revealed the highest differential expression level, with 3979 genes. These upregulated genes included SALL2 and DPYSL5 in the Apatinib-treated resistant line. In the present context, upregulation of SALL2 strongly suggests that this gene may have a complex intermediary role in modifying response to drugs. Based on the comparison 1 and comparison 4, the SALL2 is very much linked to the Apatinib treatment. DPYSL5 encodes a product that might be involved in neural development, axon guidance, and cell-cell communication and structure (Kato, 2024). The downregulated genes in treated PC9GRwere CDYL2 and CALML5. CDYL2 is a chromatin remodeler that modulates gene expression, and its inhibition might considerably alter gene expression profiles as a resistance mechanism. CALML5 is a calcium-binding protein involved in signal transduction and could contribute to changes in cell signaling relevant to resistance. Among the hallmark gene sets, there was significant enrichment of the G2M checkpoint and E2F targets in treated cells. The enriched KEGG pathways were cell cycle and DNA replication incells treated by Apatinib. Moreover, hypoxia, IL2 STAT5 signaling pathways, and myogenesis were enriched for Apatinib-treated PC9 cells but not for Apatinib-treated PC9GR cells, where only the aminoacyl-tRNA biosynthesis pathway was enriched.

Shared Gene Expression amongst Cell Lines: The comparison of gene expression across the different cell lines and treatments allowed for insights into locating target genes that are responsible to the drug resistance. The Venn diagram (Figure 1A) analysis shows that 616 genes were shared between PC9 versus PC9GR and PC9GR versus PC9GR with Apatinib.

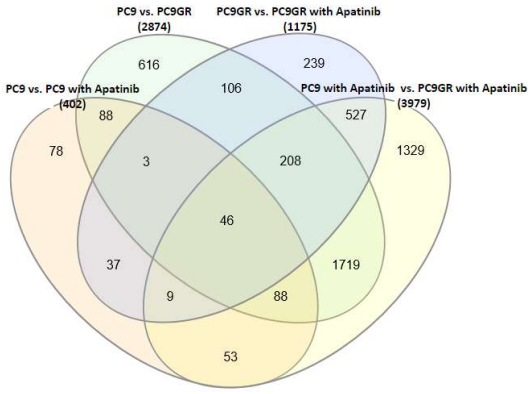

Figure 1A. Venn diagram shows the number of common and unique genes shared among different comparisons. Generated with interactiVenn

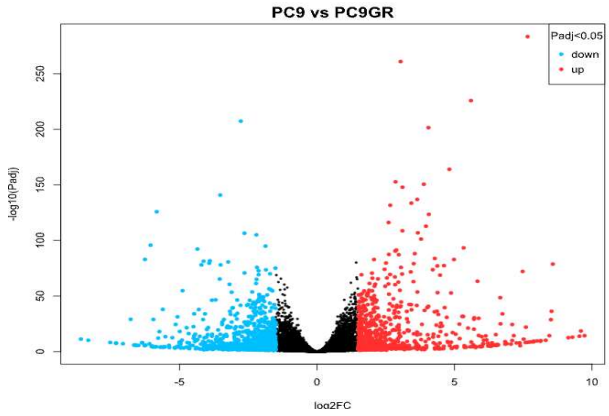

Figure 2A is the volcano plot shows upregulated and downregulated genes along with their log2foldchange and –log10(padj) value

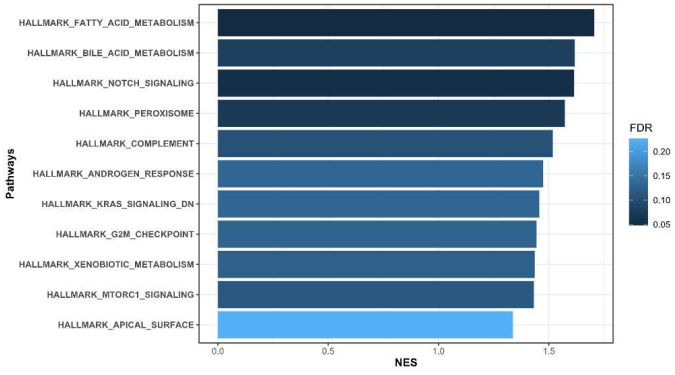

Figure 2B. Shows enriched pathways in the Hallmark gene set.

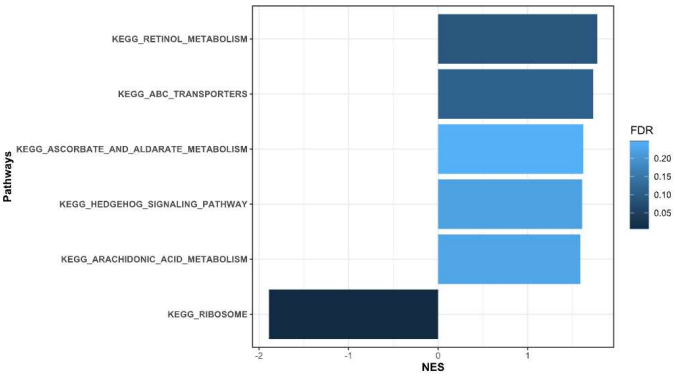

Figure 2C. Shows enriched pathways in the KEGG gene set

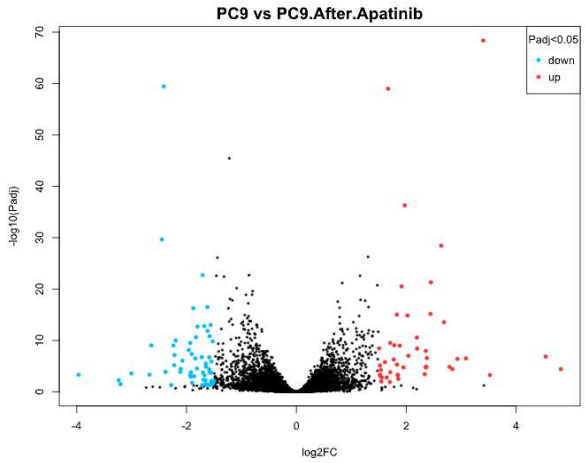

Figure 3A is the volcano plot shows upregulated and downregulated genes along with their log2foldchange and –log10(padj) value

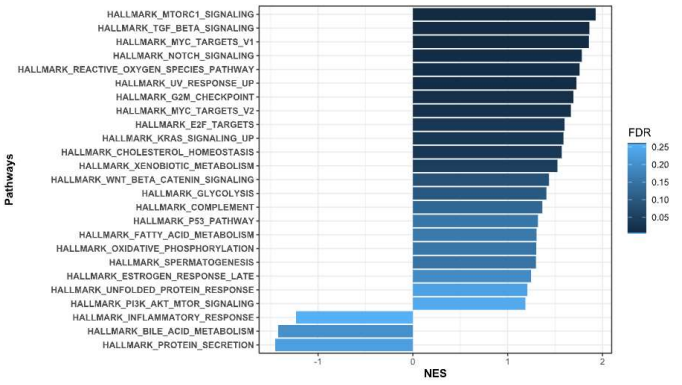

Figure 3B. Shows enriched pathways in the Hallmark gene set

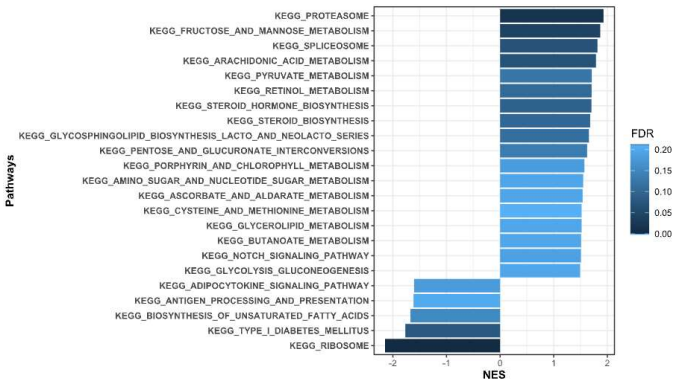

Figure 3C. shows enriched pathways in the KEGG gene set

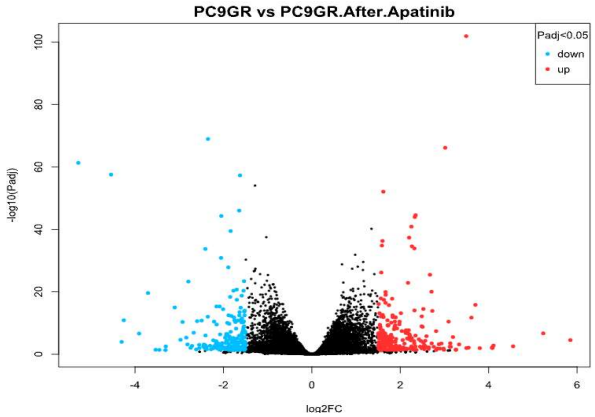

Figure 4. A is the volcano plot shows upregulated and downregulated genes along with their log2foldchange and –log10(padj) value

Table 1. Table shows the 5 most upregulated/downregulated gene names from all 4 comparisons, along with their pvalue, padj, and log 2 fold change values

Comparison Type	Gene Symbol	P-value	Padj	log2foldchange	Regulation Type
PC9 vs PC9GR	ARHGAP28	4.55E-16	7.89E-15	9.725165	Upregulated
PC9 vs PC9GR	TLR4	1.28E-20	3.4E-19	9.591799	Upregulated
PC9 vs PC9GR	GALC	1.38E-15	2.28E-14	9.538978	Upregulated
PC9 vs PC9GR	ZNF160	1.24E-14	1.86E-13	9.282516	Upregulated
PC9 vs PC9GR	LINC01060	3.91E-14	5.64E-13	9.134849	Upregulated
PC9 vs PC9GR	SALL2	7.18E-13	9.07E-12	-8.59752	Downregulated
PC9 vs PC9GR	MAP9	1.07E-11	1.18E-10	-8.32727	Downregulated
PC9 vs PC9GR	LOC105373903	1.54E-09	1.28E-08	-7.53124	Downregulated
PC9 vs PC9GR	LINC02689	5.94E-09	4.57E-08	-7.31806	Downregulated
PC9 vs PC9GR	ZNF714	6.31E-09	4.84E-08	-7.31804	Downregulated
PC9 vs PC9 with Apatinib	FAM30A	0.00463	0.0325	4.812307	Upregulated
PC9 vs PC9 with Apatinib	CYP26A1	5.47E-08	0.00000246	4.530385	Upregulated
PC9 vs PC9 with Apatinib	TRIM31	2.21E-77	3.49E-73	3.395097	Upregulated
PC9 vs PC9 with Apatinib	SERPINB3	1.22E-09	7.56E-08	3.079951	Upregulated
PC9 vs PC9 with Apatinib	TCN1	8.32E-10	5.36E-08	2.924799	Upregulated
PC9 vs PC9 with Apatinib	PRSS2	0.000323	0.00399	-3.23865	Downregulated
PC9 vs PC9 with Apatinib	HCG26	0.0000675	NA	-3.01255	Downregulated
PC9 vs PC9 with Apatinib	AFDN-DT	0.000318	NA	-2.68203	Downregulated
PC9 vs PC9 with Apatinib	LOC107985349	7.16E-12	7.29E-10	-2.64681	Downregulated
PC9 vs PC9 with Apatinib	CARMIL2	0.0018	NA	-2.62201	Downregulated
PC9GR vs PC9GR with Apatinib	DIO2-AS1	6.18E-10	1.92E-08	5.25	Upregulated
PC9GR vs PC9GR with Apatinib	SAMD15	8.11E-15	7.67E-13	3.72	Upregulated
PC9GR vs PC9GR with Apatinib	DIO2	1.38E-10	4.87E-09	3.63	Upregulated
PC9GR vs PC9GR with Apatinib	TXNIP	3.14E-181	5.33E-177	3.51	Upregulated
PC9GR vs PC9GR with Apatinib	PLAC8	0.0000372	0.00032	3.35	Upregulated
PC9GR vs PC9GR with Apatinib	HSPA6	2.31E-71	7.85E-68	-5.27	Downregulated
PC9GR vs PC9GR with Apatinib	CYP26A1	7.98E-65	2.26E-61	-4.52	Downregulated
PC9GR vs PC9GR with Apatinib	FAM30A	1.27E-13	9.07E-12	-4.28	Downregulated
PC9GR vs PC9GR with Apatinib	HSPA7	1.83E-14	1.56E-12	-4.24	Downregulated
PC9GR vs PC9GR with Apatinib	KLF15	7.06E-09	0.000000167	-3.89	Downregulated
PC9 with Apatinib vs PC9GR with Apatinib	SALL2	1.57E-15	1.27E-14	9.537972	Upregulated
PC9 with Apatinib vs PC9GR with Apatinib	DPYSL5	1.33E-14	1E-13	9.2616	Upregulated
PC9 with Apatinib vs PC9GR with Apatinib	HSPA6	1.2E-30	2.65E-29	8.52825	Upregulated
PC9 with Apatinib vs PC9GR with Apatinib	CYP26A1	3.78E-28	7.11E-27	8.115528	Upregulated
PC9 with Apatinib vs PC9GR with Apatinib	LOC105373903	6.22E-10	3.09E-09	7.655008	Upregulated
PC9 with Apatinib vs PC9GR with Apatinib	CDYL2	4.41E-21	5.44E-20	-11.1758	Downregulated
PC9 with Apatinib vs PC9GR with Apatinib	CALML5	1.94E-16	1.7E-15	-9.82404	Downregulated
PC9 with Apatinib vs PC9GR with Apatinib	PRSS2	7.2E-12	4.33E-11	-9.41862	Downregulated
PC9 with Apatinib vs PC9GR with Apatinib	TLR4	3.44E-18	3.42E-17	-8.99403	Downregulated
PC9 with Apatinib vs PC9GR with Apatinib	MUC5B-AS1	2.01E-13	1.37E-12	-8.93168	Downregulated

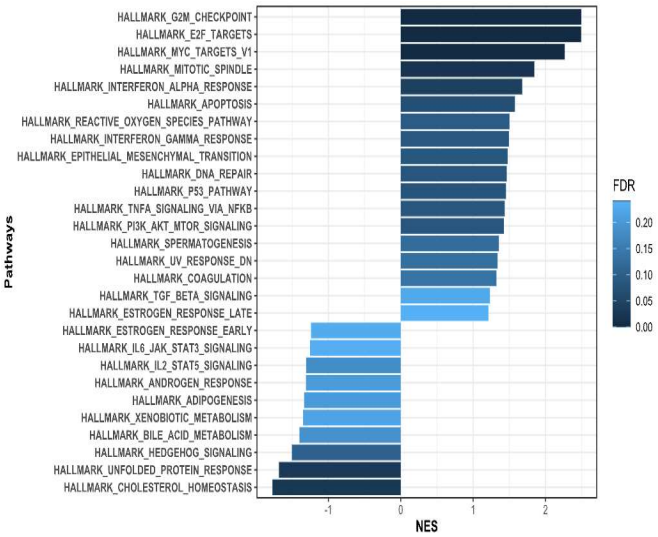

Figure 4B. Shows enriched pathways in the Hallmark gene set

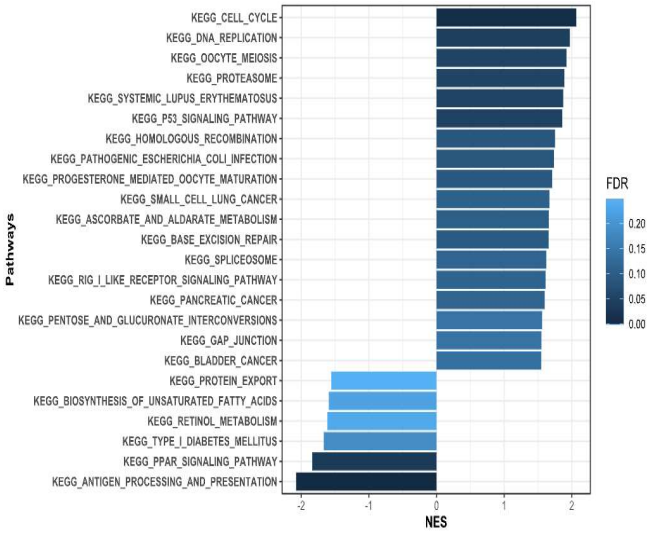

Figure 4C. Shows enriched pathways in the KEGG gene set

This means these genes are implicated in the acquisition of resistance to transition, and their activity was further modified with Apatinib treatment. Moreover, 239 genes overlapped between PC9GR vs. PC9GR with Apatinib and PC9 with Apatinib vs. PC9GR with Apatinib, indicating that these genes might be responsible for the response of Apatinib in both sensitive and resistant cells. The intersection of 527 genes between PC9 versus PC9GR and PC9 with Apatinib versus PC9GR with Apatinib marked the essential genes participating in conveying resistance development and differential drug response. Furthermore, 208 genes were reciprocally skewered in PC9 vs. PC9GR, PC9GR vs. PC9GR with Apatinib, and PC9 with Apatinib vs. PC9GR with Apatinib, underlining their role in the mechanisms of resistance and drug response. Moreover, 106 genes were shared among PC9 vs. PC9GR, PC9 vs. PC9 with Apatinib, and PC9 with Apatinib vs. PC9GR with Apatinib, representing the everyday genetic events in resistance development and primary

treatment with drugs. An even smaller subset of 78 genes was found to overlap between PC9 vs. PC9 with Apatinib and PC9GR vs. PC9GR with Apatinib, indicating genes involved in the immediate response to Apatinib in both sensitive and resistant states.

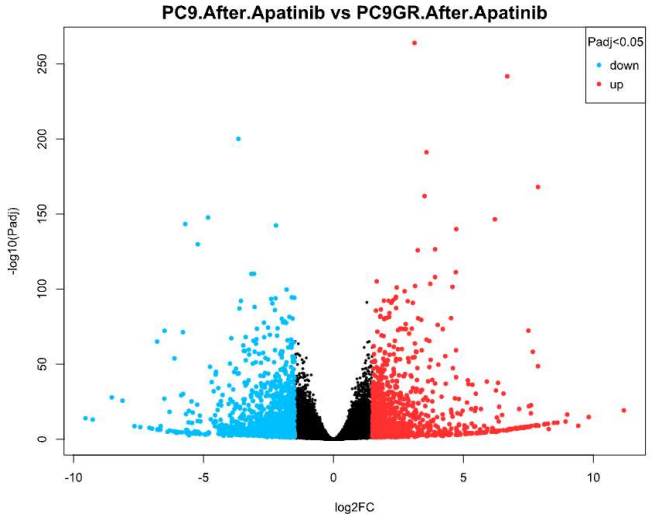

Figure 5A. is the volcano plot shows upregulated and downregulated genes along with their log2foldchange and –log10(padj) value

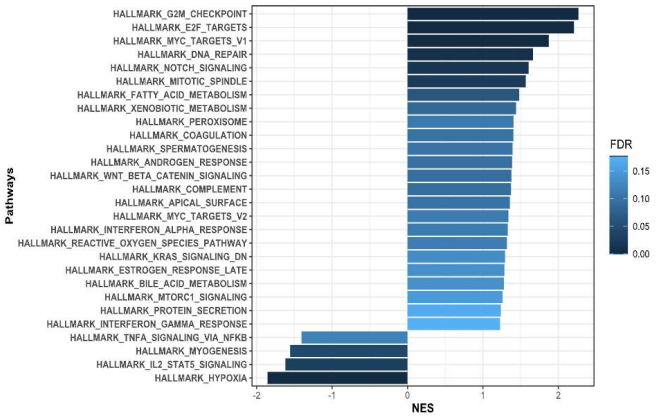

Figure 5B. Shows enriched pathways in the Hallmark gene set

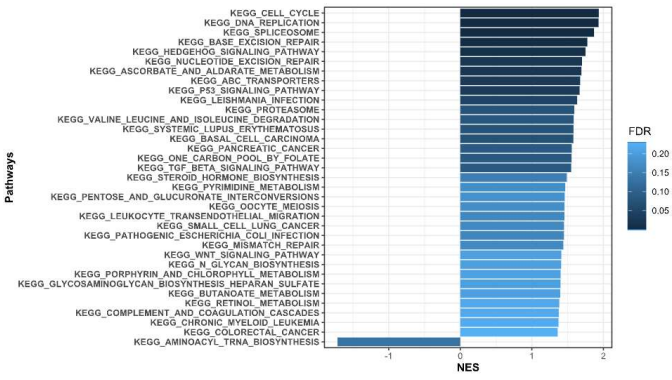

Figure 5C. Shows enriched pathways in the KEGG gene set

A still smaller overlap of 9 genes, between PC9 vs. PC9 with Apatinib and PC9 with Apatinib vs. PC9GR with Apatinib, highlighted a minimal typical response to Apatinib, irrespective of resistance status. Moreover, 46 genes were commonly shared among PC9 vs. PC9GR, PC9 vs. PC9 with Apatinib, and PC9 with Apatinib vs. PC9GR with Apatinib, indicating they are essential during resistance and drug response. Of particular note, three genes were common to all four comparisons; therefore, these could be critical genes that are universally involved in resistance and responses to Apatinib. This in-depth shared DEG analysis emphasizes the complexity of resistant mechanisms and multifaceted responses to Apatinib treatment, thus pointing out potential targets for developing strategies to improve the efficacy of Apatinib against NSCLC by overcoming the resistance. Specifically, this study delved into the molecular mechanisms underlying resistance to Apatinib in non-small cell lung cancer (NSCLC), focusing on differential gene expression and significant pathways in sensitive and Gefitinib-resistant PC9 cells (PC9 and PC9GR) and their respective Apatinib-treated counterparts. It highlighted the responsive mechanisms in relation to the Apatinib treatment and resistance. It is worth noting that nine genes (SALL2, CYP26A1, HSPA6, PRSS2, LOC105373903, TLR4, ARHGAP28, DPYSL5 and CDYL2) shared between PC9 vs. PC9 with Apatinib and PC9 with Apatinib vs. PC9GR with Apatinib. More significantly, there are three genes (SALL2, CYP26A1 and HSPA6) commonly identified in all four comparisons in this study, indicating their universal roles in Apatinib resistance. It is possible that these genes are central to understanding the development of drug resistance and response to Apatinib, serving as novel universal biomarkers or therapeutic targets with less attention in former research.

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