RNA-seq–Based Mechanistic Evaluation of Phytochemicals and Small Molecules in Experimental Cancer Models

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Abstract

High-throughput RNA sequencing (RNA-seq) has emerged as a powerful tool for deciphering the molecular mechanisms underlying phytochemical and small molecule-mediated cancer suppression. This comprehensive review synthesizes current knowledge on integrating RNA-seq-derived transcriptomic analysis with mechanistic evaluation of phytochemical and synthetic compound effects on apoptosis, inflammation, oxidative stress, and epithelial-mesenchymal transition (EMT) in experimental cancer models. We examine how transcriptomic profiling links gene expression changes to known biochemical and cellular mechanisms of action, with particular emphasis on major phytochemicals including curcumin, resveratrol, and sulforaphane. Integration of pathway enrichment analyses using KEGG (Kyoto Encyclopedia of Genes and Genomes) and Gene Ontology (GO) databases enables systematic identification of affected molecular pathways and biological processes. We discuss methodological approaches for connecting RNA-seq signatures to functional phenotypes, validation strategies for mechanistic hypotheses, and challenges in translating transcriptomic findings to therapeutic development. The review emphasizes how multi-level transcriptomic analysis encompassing individual gene expression, pathway enrichment, gene co-expression networks, and temporal dynamics provides comprehensive mechanistic insights superior to single-methodology approaches. Finally, we address future directions including integration of multi-omics data, spatial transcriptomics for tumor microenvironment characterization, and machine learning approaches for signature discovery and mechanism prediction.

Keywords: RNA-seq, phytochemicals, mechanism of action, apoptosis, inflammation, oxidative stress, EMT, pathway enrichment, KEGG, Gene Ontology

1. Introduction

Cancer remains a leading cause of mortality globally, and the development of effective therapeutic and chemopreventive agents continues as a critical research priority (Kumar et al., 2015). Natural products and phytochemicals have historically served as sources of therapeutic compounds, with curcumin, resveratrol, and sulforaphane representing well-characterized examples demonstrating potent anticancer activity in preclinical studies (Saleh et al., 2021). However, comprehensive understanding of the molecular mechanisms underlying their biological effects remains challenging, particularly when compounds target multiple pathways simultaneously (Tran et al., 2014).

Traditional approaches to mechanism elucidation employing individual qRT-PCR assays, Western blotting, or immunohistochemistry provide hypothesis-driven validation of suspected targets but lack the unbiased, genome-

wide perspective necessary for identifying unexpected pathway involvement or novel therapeutic targets (Balusamy et al., 2019). RNA sequencing (RNA-seq) fundamentally addresses these limitations by providing comprehensive, quantitative assessment of transcriptional changes across the entire transcriptome, enabling discovery-driven mechanistic insights (Balusamy et al., 2019).

The integration of RNA-seq with functional assays and pathway analysis represents a paradigm shift in cancer mechanism research, permitting researchers to simultaneously: (1) identify genes differentially expressed in response to phytochemical or small molecule treatment, (2) contextualize these changes within known biological pathways and processes, (3) validate mechanistic hypotheses suggested by transcriptomic alterations, and (4) discover novel targets and mechanisms unsuspeced from prior literature (Balusamy et al., 2019). This systems-level approach transcends traditional reductionist methodology, instead embracing the inherent complexity of biological responses to chemical perturbation (Singh et al., 2022).

Four critical cellular processes frequently targeted by anticancer phytochemicals apoptosis, inflammation, oxidative stress, and epithelial-mesenchymal transition (EMT) represent the focus of this review. Each process involves coordinated regulation of multiple genes encoding enzymes, transcription factors, structural proteins, and signaling mediators (Zheng et al., 2023; Qi et al., 2023). RNA-seq analysis enables comprehensive characterization of how phytochemicals modulate these gene networks, often revealing that apparently independent mechanisms actually represent converging branches of integrated biological responses (Tyler et al., 2021).

This review synthesizes current knowledge on applying RNA-seq to mechanistic evaluation of phytochemicals and small molecules in cancer models, with emphasis on linking transcriptomic signatures to functional phenotypes through pathway enrichment analysis, gene co-expression networks, and temporal dynamics. We examine how KEGG pathway analysis and Gene Ontology annotation transform raw gene lists into actionable biological insights, discuss validation strategies for mechanistic hypotheses derived from transcriptomic data, and address critical challenges in translating findings to drug development and clinical translation.

2. Apoptosis: Transcriptomic Signatures and Phytochemical-Induced Cell Death

2.1 Intrinsic Apoptotic Pathway Gene Regulation

Apoptosis represents one of the most extensively studied endpoints of phytochemical treatment in cancer models, reflecting its fundamental importance in cancer prevention and therapy (Alarifi et al., 2017; Vogler et al., 2025). The intrinsic apoptotic pathway triggered by cellular stress including DNA damage, growth factor withdrawal, and oxidative stress is critically regulated by the B cell lymphoma 2 (BCL2) family of proteins, which serve as molecular gate-keepers controlling mitochondrial outer membrane permeabilization (MOMP) and subsequent caspase activation (Vogler et al., 2025).

Pro-apoptotic BCL2 family members including BAX, BAK, BIM, and BAD are activated by cellular stress and promote mitochondrial permeabilization, leading to cytochrome c release into the cytosol (Alarifi et al., 2017; Vogler et al., 2025). Conversely, anti-apoptotic members including BCL2, BCL-XL, and MCL-1 sequester pro-apoptotic proteins, blocking mitochondrial dysfunction and apoptosis (Vogler et al., 2025). The balance between pro- and anti-apoptotic gene expression thus determines cellular susceptibility to apoptotic triggers, representing a critical node for cancer cell survival (Vogler et al., 2025).

RNA-seq analysis of phytochemical-treated cancer cells consistently reveals signature transcriptomic patterns reflecting pro-apoptotic shifts: upregulation of pro-apoptotic genes (BAX, BAK, BIM, BAD), downregulation of anti-apoptotic genes (BCL2, BCL-XL, MCL-1), and induction of caspase cascade members (caspase-3, caspase-9) (Floros et al., 2006; Kumar et al., 2015). Critically, these transcriptomic alterations correlate tightly with functional apoptosis measured by annexin V staining, caspase activity assays, or DNA laddering, validating that gene expression changes reflect genuine biochemical shifts in apoptotic capacity (Alarifi et al., 2017).

2.2 Phytochemical-Specific Apoptotic Gene Signatures

Distinct phytochemicals induce apoptosis through mechanistically overlapping yet discriminable transcriptomic signatures. Citral (a terpene from lemongrass), when applied to AGS gastric cancer cells, induced 612 differentially expressed genes including 216 upregulations and 396 downregulations (Balusamy et al., 2019). Pathway enrichment analysis identified apoptosis, cell cycle, and cell death as the most significantly affected biological processes (Balusamy et al., 2019). Critically, upregulated genes included canonical pro-apoptotic factors while downregulated genes encompassed anti-apoptotic mediators and cell cycle progression genes, establishing a coherent transcriptomic signature supporting apoptotic induction (Balusamy et al., 2019).

Combination treatment with resveratrol and sulforaphane in glioma cells demonstrated additive effects on apoptotic gene expression, with both agents downregulating proliferation markers (PCNA, cyclin D1) and upregulating active caspase-3 expression (Jiang et al., 2009). Transcriptomic analysis revealed that the combination treatment achieved greater suppression of pro-survival signaling protein Akt and enhanced mitochondrial dysfunction markers compared to individual agents, explaining the synergistic apoptotic phenotype (Jiang et al., 2009).

2.3 Transcriptomic Validation of Death Receptor Pathway Activation

Beyond intrinsic mitochondrial pathways, phytochemicals frequently activate extrinsic apoptosis through death receptors (FAS, TNF receptor superfamily members) and downstream adaptor molecules (FADD, caspase-8) (Kumar et al., 2015). RNA-seq analysis of death receptor pathway genes enables identification of compounds engaging this parallel apoptotic mechanism. Upregulation of death receptor ligands (TNF-α, FAS ligand, TRAIL) coupled with receptor expression changes provides transcriptomic evidence for extrinsic pathway activation, complementing functional assessments of death receptor signaling (Kumar et al., 2015).

3. Inflammatory Pathways: NF-κB Regulation and Cytokine Gene Expression

3.1 NF-κB Pathway Architecture and Transcriptional Control

Nuclear Factor-κB (NF-κB) represents one of the most frequently dysregulated transcription factors in cancer, functioning as a central hub linking inflammation to tumor promotion, progression, and immune evasion (Deshmukh et al., 2021; Banerjee & Ghosh, 2017). The canonical NF-κB pathway involves stimulus-dependent phosphorylation and degradation of inhibitory proteins (IκBα), liberating NF-κB heterodimers (typically p65/p50) for nuclear translocation and DNA binding at κB response elements in target gene promoters (Banerjee & Ghosh, 2017).

Critical NF-κB target genes encode pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, IL-17α), enzymes generating inflammatory mediators (COX-2, iNOS), chemokines (IL-8, MCP-1), and anti-apoptotic proteins (BCL2, BCL-XL, survivin) (Banerjee & Ghosh, 2017; Mahfouz et al., 2023). Phytochemicals targeting NF-κB suppress this entire gene network, producing a coordinated anti-inflammatory transcriptomic signature (Banerjee & Ghosh, 2017).

3.2 Phytochemical Inhibition of NF-κB: Molecular Mechanisms Revealed by RNA-seq

Multiple mechanistically distinct phytochemicals suppress NF-κB signaling through different molecular interventions, a distinction clarified through transcriptomic analysis. Sulforaphane, an isothiocyanate from cruciferous vegetables, demonstrates particularly potent NF-κB suppression compared to resveratrol or curcumin when applied to lipopolysaccharide/interferon-gamma-stimulated macrophages (Saleh et al., 2021). Transcriptomic analysis revealed that sulforaphane achieved 85% attenuation of induced miR-146a expression and 40% suppression of miR-155, both negative regulators of NF-κB signaling (Saleh et al., 2021).

At the mRNA level, sulforaphane produced significantly greater suppression of TNF-α and IL-6 transcription compared to equimolar concentrations of resveratrol or curcumin, despite all three agents targeting related mechanisms (Saleh et al., 2021). This differential efficacy, revealed through comparative transcriptomic analysis,

suggests distinct NF-κB pathway dependencies or parallel mechanism involvement that escaped detection in non-genome-wide approaches (Saleh et al., 2021).

Resveratrol suppresses NF-κB through SIRT1-mediated deacetylation, which the compound activates through unknown mechanisms (Tyler et al., 2021). This mechanistic complexity involving SIRT1 activation as an intermediate step upstream of NF-κB inhibition is difficult to infer from phenotypic observations alone but becomes apparent in transcriptomic signatures showing both SIRT1 target gene upregulation and NF-κB target downregulation (Tyler et al., 2021).

3.3 Inflammatory Gene Signatures and Tumor Microenvironment

Beyond direct suppression of inflammatory mediators, phytochemical-induced changes in cytokine and chemokine gene expression alter recruitment and activation of immune cells within the tumor microenvironment (Mahfouz et al., 2023). RNA-seq analysis of bulk tumors reveals complex changes in immune cell recruitment signatures reflected in altered expression of chemotactic factors, which manifest as modified immune composition detectable by deconvolution algorithms or complementary immune profiling (Mahfouz et al., 2023).

4. Oxidative Stress: Antioxidant Gene Expression and ROS-Responsive Pathways

4.1 Oxidative Stress-Related Gene Signatures

Reactive oxygen species (ROS) production and oxidative stress represent critical drivers of both cancer promotion and cancer cell death, creating a paradoxical relationship where ROS simultaneously promotes transformation while rendering cancer cells vulnerable to oxidative stress-inducing agents (Singh et al., 2022; Leone et al., 2017). Phytochemicals modulate this balance through coordinated regulation of antioxidant enzymes, ROS-generating enzymes, and ROS-responsive transcription factors, with RNA-seq enabling comprehensive characterization of these multi-faceted responses (Singh et al., 2022; Leone et al., 2017).

Oxidative stress response signatures compiled from genes encoding superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione S-transferases (GST), NAD(P)H quinone oxidoreductase 1 (NQO1), and related detoxification enzymes reveal cellular antioxidant capacity (Zheng et al., 2023). Single-cell RNA-seq analysis of ovarian cancers identified 151 genes significantly correlated with oxidative stress response, with 9 genes sufficient to construct a risk score model predictive of patient prognosis and immunotherapy response (Zheng et al., 2023).

4.2 NRF2-ARE Pathway Activation and Antioxidant Gene Expression

Nuclear factor erythroid 2-related factor 2 (NRF2) represents the master transcription factor governing antioxidant and detoxification gene expression, binding antioxidant response elements (AREs) in gene promoters under oxidative stress (Singh et al., 2022; Leone et al., 2017). Many phytochemicals directly activate NRF2 through cysteine modification of its inhibitor KEAP1, resulting in ARE-driven transcription of NQO1, GST family members, heme oxygenase-1 (HO-1), and related genes (Singh et al., 2022).

RNA-seq analysis of phytochemical-treated cells reveals robust NRF2 target gene upregulation coupled with reduced ROS levels, validating pathway activation at the transcriptomic level (Singh et al., 2022; Leone et al., 2017). Temporal RNA-seq studies show NRF2 target gene upregulation preceding ROS reduction, establishing causal relationships between transcriptional activation and functional ROS suppression (Singh et al., 2022).

4.3 ROS Generation and Metabolic Reprogramming

Paradoxically, some phytochemicals induce cancer cell death through direct ROS generation rather than antioxidant activation, a mechanistic distinction detectable through transcriptomic analysis (Shi et al., 2012). ROS-generating agents produce distinct transcriptomic signatures characterized by upregulation of ROS-responsive genes (including

p53 target genes, ATF3, early growth response factors) without concordant antioxidant gene induction (Shi et al., 2012; Leone et al., 2017).

Additionally, ROS level alterations trigger metabolic reprogramming toward alternative energy pathways, reflected in altered expression of glycolytic enzymes, pentose phosphate pathway genes, and mitochondrial respiration components (Leone et al., 2017; Shi et al., 2012). RNA-seq analysis revealing these metabolic gene expression shifts provides mechanistic insight into cellular adaptation to oxidative stress (Leone et al., 2017).

5. Epithelial-Mesenchymal Transition: Gene Networks Regulating Cell Differentiation and Invasion

5.1 EMT Transcriptional Regulatory Network

Epithelial-mesenchymal transition (EMT) represents a critical developmental program co-opted by cancer cells to promote metastasis, stemness, and therapeutic resistance (Deshmukh et al., 2021; Tyler et al., 2021). EMT involves coordinated transcriptional silencing of epithelial genes (E-cadherin, occludin, claudins) and activation of mesenchymal genes (vimentin, N-cadherin, fibronectin, COL5A2, FAP) through master transcriptional regulators including SNAIL, SLUG, TWIST, and ZEB family members (Deshmukh et al., 2021; Tyler et al., 2021).

Integrated single-cell RNA-seq and mathematical modeling revealed that TGF-β-induced EMT involves simultaneous activation of multiple signaling cascades including TGF-β/SMAD, BMP, Wnt/β-catenin, NOTCH, and Sonic Hedgehog pathways that converge on core EMT transcription factors (Deshmukh et al., 2021). This multi-pathway convergence explains why inhibition of single pathways frequently fails to suppress EMT, a complexity revealed only through genome-wide transcriptomic analysis (Deshmukh et al., 2021).

5.2 Phytochemical-Induced EMT Suppression: Molecular Mechanisms

Resveratrol suppresses TGF-β-induced EMT in colorectal cancer cells through multiple coordinated mechanisms revealed by RNA-seq analysis (Tyler et al., 2021). Treatment resulted in downregulation of mesenchymal markers (vimentin, N-cadherin, fibronectin) coupled with restoration of epithelial markers (E-cadherin), indicating phenotypic reversal (Tyler et al., 2021). Pathway enrichment analysis implicated suppression of TGF-β/SMAD signaling and Wnt/β-catenin pathways, identifying distinct upstream targets of resveratrol's EMT-suppressive activity (Tyler et al., 2021).

Additionally, resveratrol suppressed EMT-transcription factors SNAIL and SLUG while upregulating their negative regulators, establishing a multi-level suppression of EMT regulatory networks (Tyler et al., 2021). These insights that resveratrol targets multiple nodal points in EMT networks rather than sole EMT factors emerge from transcriptomic analysis but would remain cryptic in hypothesis-driven approaches focused on individual genes (Tyler et al., 2021).

5.3 Distinguishing Tumor Cell EMT from Stromal Fibroblast Signatures

A critical methodological consideration in interpreting bulk tumor RNA-seq involves distinguishing genuine EMT in cancer cells from mesenchymal gene expression in cancer-associated fibroblasts (CAFs) and other stromal components (Tyler et al., 2021). Single-cell RNA-seq studies revealed that mesenchymal signature genes are predominantly expressed in CAFs rather than cancer cells themselves, suggesting that apparent bulk-level EMT signatures often reflect stromal composition changes rather than cancer cell EMT (Tyler et al., 2021).

Deconvolution approaches using reference single-cell RNA-seq data enable computational separation of cancer cell-specific vs stromal EMT signatures in bulk transcriptomic data, providing more accurate mechanistic insights into whether phytochemicals directly suppress cancer cell EMT or primarily alter stromal composition (Tyler et al., 2021). This methodological refinement demonstrates how advanced transcriptomic analysis prevents mechanistic misinterpretation (Tyler et al., 2021).

6. Pathway Enrichment Analysis: From Gene Lists to Mechanistic Insights

6.1 Gene Ontology Annotation and Biological Process Identification

Gene Ontology (GO) provides hierarchical annotation of gene function across three biological domains: molecular function (biochemical activities), cellular component (subcellular localization), and biological process (higher-level physiological processes) (Subramanian et al., 2005; Khatri et al., 2012). Enrichment analysis identifies GO terms significantly overrepresented among differentially expressed genes, revealing which biological processes and cellular functions are coordinately regulated by phytochemical treatment (Subramanian et al., 2005).

For example, enrichment analysis of citral-treated gastric cancer cells identified GO terms including "cell death," "apoptotic process," "response to stress," and "regulation of apoptosis" among upregulated genes, while downregulated genes enriched for "cell cycle," "DNA replication," and "mitotic cell cycle" terms (Balusamy et al., 2019). This GO enrichment pattern provides strong statistical validation that citral treatment coordinately activates apoptotic pathways while suppressing proliferation a mechanistic conclusion that emerges from integrated analysis rather than inspection of individual genes (Balusamy et al., 2019).

6.2 KEGG Pathway Analysis and Signaling Network Mapping

KEGG pathway analysis integrates genes into curated maps of cellular signaling cascades, metabolic pathways, and disease-associated pathways, enabling interpretation of gene expression changes at the pathway level (Kanehisa & Goto, 2000; Kanehisa et al., 2012). Sample-level KEGG pathway enrichment analysis (SLEA) generates enrichment scores for each pathway in individual tumor samples, revealing heterogeneity in pathway activation across tumors (Wanggou et al., 2016).

Glioblastoma classification based on KEGG pathway enrichment patterns identified five subtypes with distinct molecular characteristics and clinical outcomes a classification dimension invisible at individual gene or microarray platform levels (Wanggou et al., 2016). Similarly, gastric cancer pathway enrichment analysis identified ECM-receptor interaction, protein digestion and absorption, focal adhesion, and PI3K-Akt signaling as key altered pathways, with identified hub genes (COL1A2, FN1, BGN, THBS2, COL5A2, COL6A3) suggesting that extracellular matrix remodeling represents a critical cancer phenotype (Zhu et al., 2019).

6.3 Mechanistic Interpretation of Pathway Enrichment Results

Integration of GO and KEGG analyses enables layered interpretation moving from specific molecular functions to high-level biological processes and disease-associated pathways. When phytochemical treatment results in downregulation of KEGG "apoptosis" pathway genes specifically while upregulating "NF-κB signaling" and "TNF signaling" pathways, this pattern suggests the compound simultaneously promotes apoptotic machinery while inhibiting survival signaling a nuanced mechanistic portrait difficult to glean from individual gene analysis (Kanehisa & Goto, 2000; Kanehisa et al., 2012).

7. Linking Gene Expression to Functional Phenotypes: Validation Strategies

7.1 Integrated Transcriptomics and Functional Assays

While RNA-seq identifies transcriptional changes, functional validation remains essential to establish that observed gene expression alterations produce expected biochemical and cellular phenotypes (Balusamy et al., 2019). Comprehensive studies integrate RNA-seq with complementary functional assays: apoptosis detection by flow cytometry and annexin V staining, ROS measurement by fluorescent probes, migration/invasion assays, and metabolic assays (Balusamy et al., 2019).

For citral-treated AGS cells, RNA-seq identified apoptotic pathway upregulation, subsequently validated by: (1) significantly elevated annexin V-positive populations compared to control cells, (2) upregulation of active caspase-3 protein by Western blot, (3) enhanced mitochondrial membrane depolarization, and (4) DNA laddering characteristic of apoptosis (Balusamy et al., 2019). This multi-method validation anchors transcriptomic inferences to physiological reality (Balusamy et al., 2019).

7.2 Temporal RNA-seq Studies and Mechanistic Sequencing

Mechanistic understanding benefits from temporal RNA-seq analysis capturing how gene expression changes evolve during phytochemical treatment, establishing cause-and-effect relationships within signaling cascades (Balusamy et al., 2019). Early-response genes (transcription factors, immediate early genes, signal transduction molecules) whose upregulation precedes later changes in effector molecules (apoptotic proteins, metabolic enzymes) suggest regulatory relationships (Balusamy et al., 2019).

For example, if resveratrol treatment shows SIRT1 upregulation at 2 hours preceding NF-κB target gene downregulation at 4-6 hours, transcriptomic temporal analysis suggests SIRT1 represents an upstream regulator of NF-κB suppression, a causal relationship distinguishable from mere correlation through time-course data (Jiang et al., 2009).

7.3 Mechanistic Hypothesis Generation from Co-Expression Networks

Weighted gene co-expression network analysis (WGCNA) identifies modules of highly correlated genes whose expression changes coordinately during phytochemical treatment (Zhang et al., 2018). Hub genes within co-expression modules genes with the highest connectivity to other module members represent candidates for mechanistic investigation, as they may represent regulatory nodes controlling module-wide expression (Zhang et al., 2018).

If WGCNA identifies a co-expression module upregulated by phytochemical treatment containing multiple NRF2 target genes with NRF2 as the highest-connectivity hub gene, this result provides computational evidence supporting NRF2 as a key mechanistic regulator, guiding hypothesis-driven functional validation (Zhang et al., 2018).

8. Small Molecule Mechanistic Evaluation: Case Studies in Targeted and Multi-Target Inhibition

8.1 Selective vs Promiscuous Transcriptional Effects

Small molecule inhibitors demonstrate variable degrees of selectivity for intended targets, with RNA-seq enabling comprehensive assessment of off-target effects and unintended transcriptional consequences (Tran et al., 2014; Huang et al., 2017). Stauprimide, a selective small molecule suppressing MYC transcription, demonstrated remarkable specificity when assessed by mRNA-seq and gene set enrichment analysis (Bouvard et al., 2017). Among 50 hallmark gene sets queried, only MYC target gene sets showed statistically significant enrichment during stauprimide treatment, indicating selective MYC suppression without broad transcriptional disruption (Bouvard et al., 2017).

In contrast, staurosporine a protein kinase inhibitor produces widespread transcriptional changes affecting numerous gene sets, reflecting its promiscuous kinase inhibition (Bouvard et al., 2017; Tran et al., 2014). Comprehensive transcriptomic analysis thus distinguishes truly selective compounds from promiscuous ones, informing development strategies favoring selectivity (Tran et al., 2014).

8.2 Cancer Cell-Specific vs Pleiotropic Gene Expression Changes

Small molecules frequently produce distinct gene expression patterns in cancer vs normal cells, reflecting differential genetic backgrounds, oncogenic pathway dependencies, and metabolic reprogramming in transformed cells (Tran et

al., 2014). RNA-seq analysis of matched cancer/normal cell pairs revealed that experimental compounds induced 311 genes upregulated 3-fold or more in cancer cells, while only 12 genes showed similar upregulation in matched normal cells, indicating cancer selectivity at the transcriptomic level (Tran et al., 2014).

This selectivity, revealed through comparative transcriptomic analysis, provides mechanistic insight into cancer selectivity emerging from genomic or epigenetic differences between cell types (Tran et al., 2014). Compounds inducing cancer-specific transcriptional changes frequently demonstrate enhanced therapeutic activity, as determined gene expression normalization in cancer cells without toxicity to normal cells (Tran et al., 2014).

8.3 Kinase Inhibitor Signature Gene Profiles

Comprehensive transcriptomic analysis of kinase inhibitors revealed shared signature gene expression patterns across diverse kinase inhibitor classes, distinguishing this inhibitor class from other anticancer agent categories (Mashima et al., 2015). Gene ontology analysis of genes commonly altered by kinase inhibitors identified enrichment in transcriptional regulation, apoptosis, and cell cycle categories biological processes downstream of multiple kinase-dependent pathways (Mashima et al., 2015).

This signature pattern enables rapid classification of novel compounds with kinase inhibitory activity through transcriptomic profiling, even without prior knowledge of specific kinase targets (Mashima et al., 2015). Conversely, deviations from expected kinase inhibitor signatures suggest novel mechanistic properties worthy of investigation (Mashima et al., 2015).

9. Single-Cell RNA-seq and Cellular Heterogeneity in Mechanistic Responses

9.1 Cell Type-Specific Apoptotic Responses

Bulk RNA-seq obscures heterogeneous responses to phytochemical treatment across cell types within tumors and cell cultures (Deshmukh et al., 2021; Tyler et al., 2021). Single-cell RNA-seq reveals that phytochemical-induced apoptotic gene expression changes may be restricted to specific cell populations (cancer cells, particular immune infiltrates, endothelial cells) while others show minimal response (Deshmukh et al., 2021).

For example, TGF-β-induced EMT in single-cell RNA-seq data exhibited progressive pseudotime trajectories where subsets of cells exhibited complete EMT profiles while others showed partial EMT or retained epithelial phenotypes (Deshmukh et al., 2021). This cellular heterogeneity invisible in bulk RNA-seq explains variable phytochemical responses and suggests that combination approaches addressing multiple cell states may enhance efficacy (Deshmukh et al., 2021).

9.2 Tumor Microenvironment Composition Changes

Single-cell RNA-seq enables identification of altered immune infiltration and stromal composition following phytochemical treatment, distinguishing direct effects on cancer cells from immune-mediated anti-tumor effects (Tyler et al., 2021). Enhanced recruitment of antitumor immune cells (CD8+ T cells, M1-polarized macrophages) or reduced abundance of immunosuppressive populations (regulatory T cells, myeloid-derived suppressor cells) detected through scRNA-seq signatures reveal mechanistic contributions from immune activation (Tyler et al., 2021).

10. Temporal Dynamics and Dose-Response Transcriptomic Analysis

10.1 Early-Response vs Late-Response Gene Expression Programs

Temporal RNA-seq experiments capturing transcriptional changes at multiple timepoints reveal that phytochemical-induced gene expression follows characteristic temporal patterns: early transient changes in signal transduction and stress response genes, followed by sustained alterations in effector molecules and metabolic genes (Balusamy et al.,

2019). This temporal structure provides mechanistic insight into pathway hierarchy and causality (Balusamy et al., 2019).

Early upregulation of ATF3, Jun, Fos, and other immediate early genes suggests direct phytochemical sensing or primary stress response, while delayed upregulation of caspases and apoptotic genes indicates downstream consequences of early events (Balusamy et al., 2019). Temporal ordering effectively establishes regulatory relationships in signaling cascades (Balusamy et al., 2019).

10.2 Dose-Dependent Gene Expression Changes

Dose-response RNA-seq experiments quantify transcriptional changes as phytochemical concentration varies, identifying genes showing steep dose-dependent changes (likely mechanistically central) vs genes with shallow dose-response curves (possibly secondary) (Leone et al., 2017; Singh et al., 2022). Genes with IC50 concentrations matching compound's functional IC50 (concentration inhibiting cell proliferation 50%) likely represent direct mechanistic targets (Leone et al., 2017; Singh et al., 2022).

11. Multi-Omics Integration: Moving Beyond Transcriptomics

11.1 Genomic Mutations and Transcriptomic Response

Integration of genomic mutations with transcriptomic responses reveals how genetic background influences phytochemical efficacy (Zheng et al., 2023). Tumors with specific mutations in genes encoding phytochemical targets may show attenuated transcriptomic responses, while tumors with synthetic lethal interactions may show exaggerated effects (Zheng et al., 2023). Comprehensive mutation analysis combined with transcriptomics predicts treatment response more accurately than either modality alone (Zheng et al., 2023).

11.2 Epigenetic Modifications and Gene Expression Regulation

DNA methylation and histone modification patterns influence phytochemical-induced gene expression changes by determining chromatin accessibility and transcription factor binding (Zheng et al., 2023). Integration of ATAC-seq (assay for transposase-accessible chromatin) with RNA-seq identifies genes showing treatment-induced chromatin opening and concurrent transcriptional upregulation, establishing epigenetic mechanisms underlying mechanistic responses (Zheng et al., 2023).

11.3 Proteomics Validation and Post-Translational Modifications

While RNA-seq quantifies mRNA abundance, protein abundance and post-translational modifications critically influence functional outcomes (Singh et al., 2022; Leone et al., 2017). Integration of quantitative proteomics validates transcriptomic findings and identifies instances where mRNA and protein changes diverge due to translational or post-translational regulation (Singh et al., 2022). Phosphoproteomics specifically identifies activation of kinases and other signaling proteins, essential for mechanistic understanding of pathway activation (Singh et al., 2022).

12. Challenges and Limitations in RNA-seq Mechanistic Studies

12.1 Interpretation of Correlation vs Causation

RNA-seq identifies correlations between phytochemical treatment and gene expression changes but does not inherently establish causation (Balusamy et al., 2019). Upregulated genes may represent direct targets of phytochemical-modulated transcription factors, secondary consequences of primary target modulation, or markers of off-target effects (Balusamy et al., 2019). Functional validation through loss-of-function studies (gene

knockdown, knockout) confirms causal relationships between gene expression changes and phenotypic endpoints (Balusamy et al., 2019).

12.2 Tumor Heterogeneity and Sample-to-Sample Variation

Inter-tumor transcriptional heterogeneity arising from diverse genetic backgrounds, microenvironmental contexts, and clonal composition generates substantial sample-to-sample variation that can obscure compound-induced transcriptional signals (Tyler et al., 2021; Zheng et al., 2023). Adequate sample sizes and rigorous statistical analysis accounting for biological variation are essential for reproducible mechanistic conclusions (Tyler et al., 2021).

12.3 Functional Annotation Uncertainty for Non-Canonical Genes

While canonical genes have well-characterized functions, many differentially expressed genes lack definitive functional annotation, limiting mechanistic interpretation (Deshmukh et al., 2021). Long non-coding RNAs, pseudogenes, and genes with sparse publication records remain functionally cryptic despite appearing prominently in differential expression analyses (Deshmukh et al., 2021). Functional validation using CRISPR/Cas9 screens or other high-throughput approaches helps clarify roles of poorly annotated genes (Deshmukh et al., 2021).

13. Future Perspectives and Emerging Technologies

13.1 Spatial Transcriptomics and Tissue Architecture

Spatial transcriptomics methods preserving tissue architecture while providing transcriptome-wide measurements promise to clarify how phytochemical treatment alters gene expression in specific tissue microregions critical for understanding immune infiltration, angiogenesis, and EMT induction in native tissue contexts (Tyler et al., 2021). Spatial resolution of transcriptomic changes will refine mechanistic understanding beyond what dissociated single-cell approaches reveal (Tyler et al., 2021).

13.2 AI-Driven Signature Discovery and Mechanism Prediction

Artificial intelligence and deep learning approaches trained on large transcriptomic datasets promise to identify gene expression patterns predictive of phytochemical mechanism and efficacy with improved sensitivity compared to traditional statistical methods (Leone et al., 2017). Neural network-based models may identify non-obvious gene combinations and cross-talk mechanisms that escape human-supervised analysis (Leone et al., 2017).

13.3 Live-Cell Imaging with Transcriptomic Readouts

Emerging technologies coupling transcriptomic measurements with live-cell imaging of reporter genes or fluorescent proteins enable real-time observation of gene expression changes in individual cells during phytochemical treatment, capturing both population-average trends and cellular heterogeneity (Deshmukh et al., 2021).

14. Conclusion

RNA-seq-based transcriptomic analysis has fundamentally transformed mechanistic evaluation of phytochemicals and small molecules in cancer models, enabling genome-wide discovery of targeted pathways, identification of unexpected mechanisms, and systematic linkage between gene expression changes and functional phenotypes. Integration of transcriptomic data with pathway enrichment analysis (KEGG, GO), co-expression network analysis, temporal dynamics, and complementary omics modalities provides comprehensive mechanistic insights superior to any single methodology.

The four critical cellular processes examined in this review apoptosis, inflammation, oxidative stress, and EMT represent convergence points where multiple phytochemicals and small molecules intersect, yet mechanistic details

distinguish individual compounds and reveal opportunities for rational combination approaches. Phytochemicals including curcumin, resveratrol, and sulforaphane each produce complex, multi-pathway transcriptomic signatures reflecting their evolutionary history as general stress-response molecules with broad biological activity.

Future advances will increasingly leverage spatial transcriptomics and single-cell resolution to move beyond population-level understanding toward mechanistic clarity at the cellular and tissue microregion level. Integration of genomic, epigenetic, proteomic, and metabolomic data alongside transcriptomics will reveal coordinated regulation across biological levels. However, the fundamental challenge remains translation of preclinical mechanistic insights from experimental models to human disease a challenge where rigorous transcriptomic characterization in human patient samples and clinical trials will ultimately determine the clinical relevance of laboratory discoveries.

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