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Cancer Drug Resistance

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Part B - Emerging Mechanisms & Therapeutic Innovation



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Editor's Note

Cancer therapy resistance remains a major barrier to durable clinical responses across solid tumors and hematologic malignancies. This section brings together a curated selection of studies published in Cancer Drug Resistance that focus on clinically relevant mechanisms of therapeutic failure and strategies to overcome them. The highlighted articles span antibody–drug conjugate (ADC) resistance, chemotherapy failure in colorectal cancer, targeted therapy resistance in KRAS-mutant disease, and apoptosis dysregulation in treatment-refractory tumors.

Collectively, these works emphasize how resistance emerges in direct response to therapeutic pressure in clinically meaningful contexts, including HER2-positive breast cancer, colorectal cancer, non-small cell lung cancer, and glioblastoma. Mechanistic insights into drug efflux transporters, signaling rewiring, tumor microenvironment remodeling, and lineage-specific transcriptional dependencies provide a framework for translating laboratory discoveries into improved therapeutic strategies.

By integrating pharmacological resistance mechanisms with disease-specific biology, this section underscores the evolving interface between cancer treatment and resistance biology. It highlights how understanding therapy-driven adaptation is essential for optimizing current regimens and guiding the development of next-generation anticancer agents.

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NFE2L2 and ferroptosis resistance in cancer therapy

Daolin Tang, Rui Kang

Keywords:

Cancer therapy, drug resistance, ferroptosis, NFE2L2, oxidative stress

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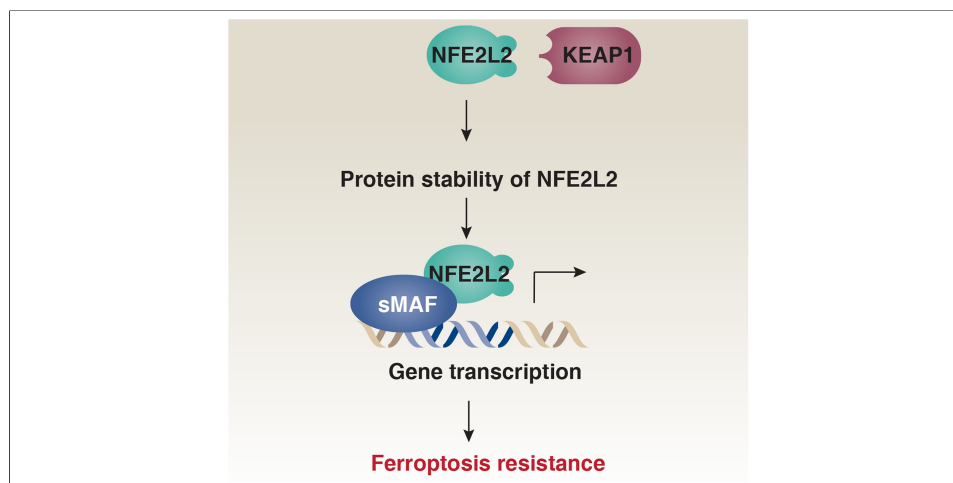
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**Abstract**

NFE2-like basic leucine zipper transcription factor 2 (NFE2L2, also known as NRF2), is a key transcription factor in the cellular defense against oxidative stress, playing a crucial role in cancer cell survival and resistance to therapies. This review outlines the current knowledge on the link between NFE2L2 and ferroptosis - a form of regulated cell death characterized by iron-dependent lipid peroxidation - within cancer cells. While NFE2L2 activation can protect normal cells from oxidative damage, its overexpression in cancer cells contributes to drug resistance by upregulating antioxidant defenses and inhibiting ferroptosis. We delve into the molecular pathways of ferroptosis, highlighting the involvement of NFE2L2 and its target genes, such as *NQO1*, *HMOX1*, *FTH1*, *FTL*, *HERC2*, *SLC40A1*, *ABCB6*, *FECH*, *PIR*, *MT1G*, *SLC7A11*, *GCL*, *GSS*, *GSR*, *GPX4*, *AIFM2*, *MGST1*, *ALDH1A1*, *ALDH3A1*, and *G6PD*, in ferroptosis resistance. Understanding the delicate balance between NFE2L2's protective and deleterious roles could pave the way for novel therapeutic strategies targeting NFE2L2 to enhance the efficacy of ferroptosis inducers in cancer therapy.

INTRODUCTION

NFE2-like basic leucine zipper transcription factor 2 (NFE2L2, also known as NRF2) is a key member of the Cap'n'Collar (CNC) family of basic leucine zipper transcription factors, which share a conserved CNC domain crucial for DNA binding



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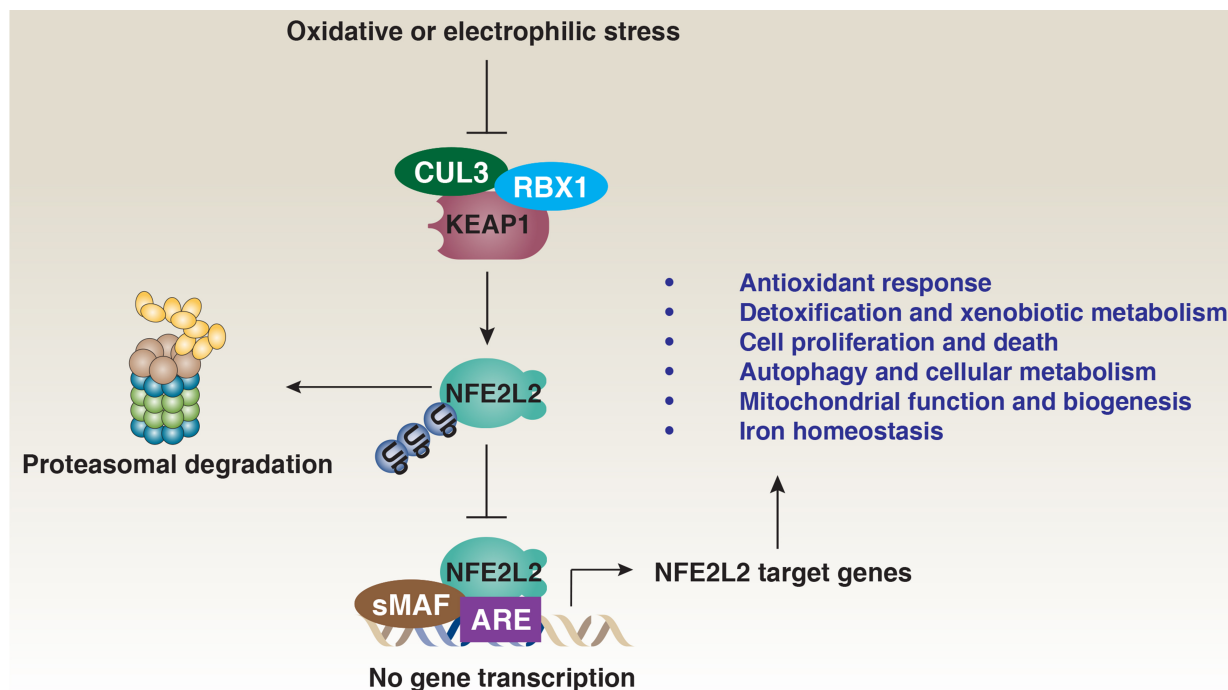


Figure 1. The KEAP1-NFE2L2 signaling pathway. NFE2L2 is primarily regulated by the KEAP1-CUL3-RBX1 E3 ubiquitin ligase complex, which, under normal conditions, promotes the ubiquitination and proteasomal degradation of NFE2L2, keeping its cellular levels low. However, in response to oxidative stress or electrophilic agents, key cysteine residues on KEAP1 are modified, preventing NFE2L2 ubiquitination. As a result, NFE2L2 stabilizes and accumulates in the cytoplasm before translocating to the nucleus. There, it dimerizes with sMAF proteins and binds to AREs in the promoters of target genes. These genes regulate a variety of crucial cellular processes, including antioxidant defense, detoxification, cell proliferation and death, autophagy, metabolism, mitochondrial function, and iron homeostasis. Through these pathways, NFE2L2-mediated gene expression plays an essential role in protecting cells from oxidative damage, maintaining redox balance, and ensuring overall cellular homeostasis. KEAP1: Kelch-like ECH-associated protein 1; NFE2L2: NFE2-like basic leucine zipper transcription factor 2; CUL3: cullin 3; RBX1: ring-box 1; sMAF: small musculoaponeurotic fibrosarcoma; AREs: antioxidant response elements.

and dimerization. NFE2L2 plays a vital role in cellular defense against oxidative stress^[1]. Under normal conditions, NFE2L2 levels are tightly controlled and kept low through rapid degradation by the ubiquitin-proteasome system [Figure 1]. Specifically, the E3 ubiquitin ligase Kelch-like ECH-associated protein 1 (KEAP1), part of the cullin 3 (CUL3)-based E3 ubiquitin ligase complex, targets NFE2L2 for degradation by binding to its Neh2 domain, which contains two KEAP1 binding sites (ETGE and DLG)^[2]. However, in response to oxidative or electrophilic stress, NFE2L2 stability increases, allowing it to accumulate in the nucleus. There, NFE2L2 dimerizes with small musculoaponeurotic fibrosarcoma (sMAF) proteins and binds to antioxidant response elements (AREs) in the promoters of target genes, leading to the expression of a wide range of detoxifying and antioxidant enzymes^[3].

NFE2L2 plays a protective role in a wide range of human diseases, including cancer, neurodegenerative disorders, cardiovascular diseases, metabolic syndromes, chronic inflammatory conditions, liver and renal diseases, and infections^[4]. In cancer, NFE2L2 exhibits a dual function depending on the disease context. In normal cells, it safeguards against oxidative damage by inducing antioxidant and detoxifying enzymes^[5]. However, in cancer cells, overactivation of NFE2L2 - often due to mutations in KEAP1 or gain-of-function mutations in NFE2L2 - leads to enhanced tumor survival by promoting antioxidant defenses, metabolic reprogramming, and resistance to oxidative stress-induced cell death, such as ferroptosis and apoptosis^[6]. This constitutive upregulation is common in cancers, including non-small cell lung cancer, pancreatic ductal

adenocarcinoma, hepatocellular carcinoma (HCC), and ovarian, bladder, endometrial, and prostate cancers^[7]. In these malignancies, elevated NFE2L2 activity supports tumor progression, metastasis, and resistance to therapies. As a result, NFE2L2 has become a key target in cancer research, with ongoing efforts to develop activators for conditions requiring enhanced oxidative defense, such as neurodegenerative diseases, and inhibitors to counteract its role in tumor resistance and poor prognosis^[8].

Ferroptosis is a form of regulated cell death characterized by the iron-dependent accumulation of lipid peroxides, which leads to oxidative damage and cell death^[9,10]. Unlike caspase-mediated apoptosis, ferroptosis is primarily driven by the failure of the cell's antioxidant systems, particularly the glutathione (GSH)-dependent glutathione peroxidase 4 (GPX4) pathway, which normally protects against lipid peroxidation^[11]. Ferroptosis has emerged as a promising target in cancer therapy, especially for treating tumors that are resistant to other forms of cell death, such as apoptosis^[12,13]. However, the development of resistance to ferroptosis presents a significant challenge to the efficacy of these therapies.

This review will first provide an overview of the molecular pathways involved in ferroptosis, followed by an in-depth discussion of how NFE2L2 is activated and contributes to drug resistance during ferroptosis-mediated cancer therapy. Understanding these mechanisms may facilitate the development of novel combination therapies designed to enhance the efficacy of ferroptosis inducers and improve anticancer outcomes.

OVERVIEW OF FERROPTOSIS PATHWAY

Ferroptosis was initially characterized as a targeted form of cell death designed to selectively eliminate cells harboring mutant *RAS* oncogenes^[14]. The discovery of ferroptosis was driven by the identification of two key compounds, erastin and RSL3, from a large compound library, both of which were found to trigger ferroptosis and inhibit tumor growth in *RAS*-mutant cells^[14,15]. Early studies highlighted the selective nature of ferroptosis in *RAS*-mutant cells, which generated interest due to its apparent safety for normal cells. However, subsequent research has demonstrated that ferroptotic damage can also occur in normal cells or tissues, implicating ferroptosis in various pathological conditions, including neurodegenerative diseases and ischemia-reperfusion injury^[16,17].

Recent findings have further complicated the role of ferroptosis in cancer therapy, suggesting that traditional ferroptosis inducers can not only kill cancer cells but may also impair antitumor immune cells, such as CD8⁺ T cells^[18,19], dendritic cells^[20], and natural killer cells^[21], thereby weakening the overall antitumor immune response. This issue arises because ferroptosis inducers such as erastin and RSL3 target solute carrier family 7 member 11 (SLC7A11) and GPX4 - proteins expressed in both normal and cancer cells. To address this challenge, a novel cell-type-specific ferroptosis inducer, N6F11, was developed to selectively target cancer cells by binding to tripartite motif-containing 25 (TRIM25) and promoting TRIM25-dependent degradation of GPX4^[22]. Because TRIM25 is primarily expressed in cancer cells and shows low or no expression in immune cells, N6F11 does not adversely affect the immune system^[22,23].

The mechanisms underlying the induction and regulation of ferroptosis have been extensively studied. Despite its plasticity and heterogeneity, ferroptosis can be induced through two major pathways: the extrinsic (transporter-dependent) pathway and the intrinsic (enzyme-regulated) pathway^[24] [Figure 2]. For example, increased transferrin receptor (TFRC)-dependent iron uptake or inhibition of the system *xc*⁻-cystine/glutamate antiporter, leading to reduced GSH production, can trigger ferroptosis^[9]. Additionally, the autophagic degradation of the iron storage protein ferritin, a process known as ferritinophagy, increases the labile iron pool and reactive oxygen species (ROS) production, thereby sensitizing cells to ferroptosis^[25,26].

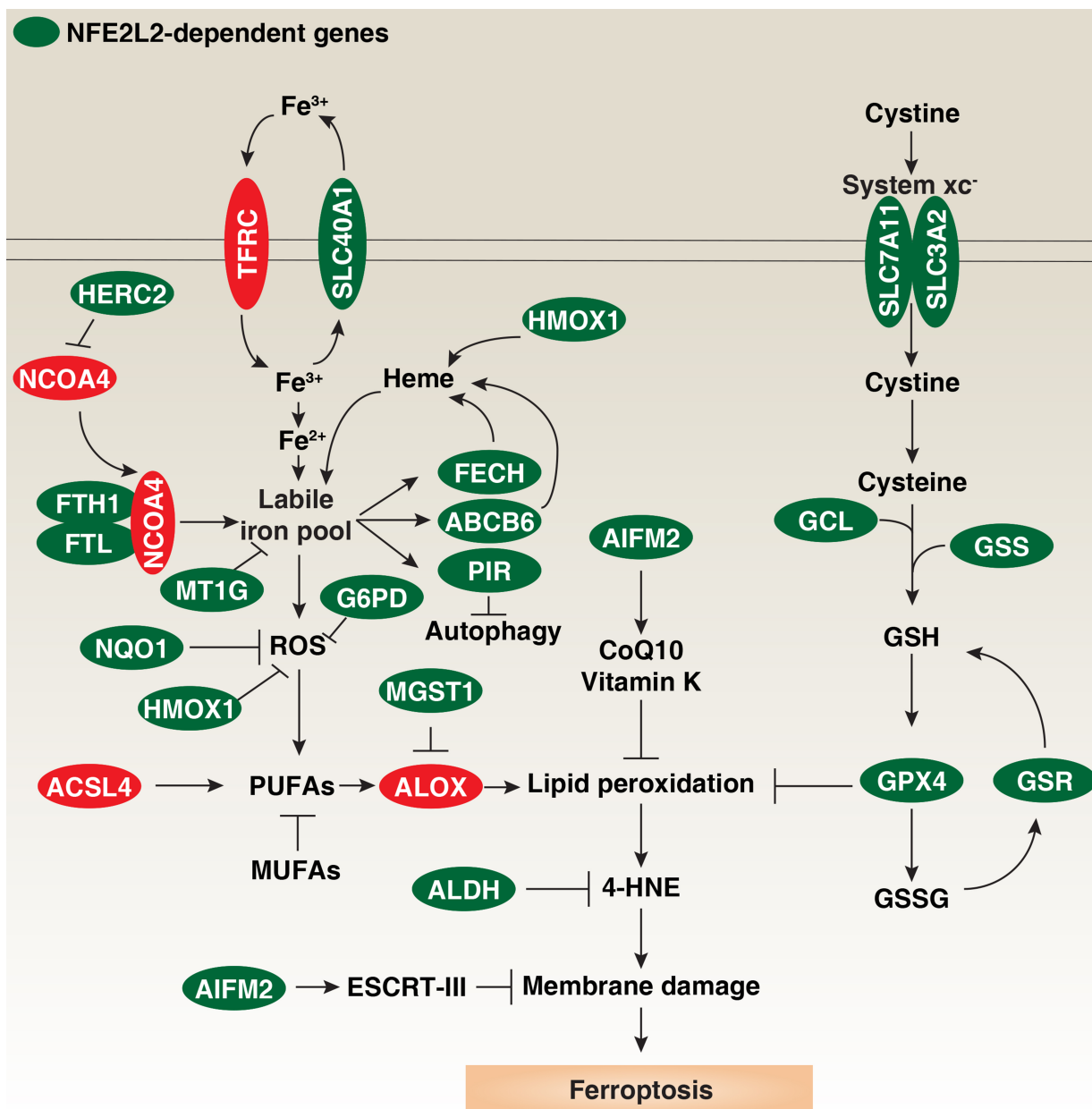


Figure 2. NFE2L2-dependent genes in ferroptosis. Ferroptosis, a form of oxidative cell death, can be triggered by increased iron accumulation or the inhibition of antioxidant systems. Fe^{3+} is taken up by TFRC and exported by SLC40A1. Inside the cell, Fe^{3+} is reduced to Fe^{2+} , with excess iron stored in ferritin (composed of FTH1 and FTL) or sequestered by MT1G. Conversely, autophagic degradation of ferritin via NCOA4 can elevate the labile iron pool, a process regulated by HERC2-mediated degradation of NCOA4 through the ubiquitin-proteasome system. HMOX1 also contributes to the labile iron pool by catalyzing heme degradation. Iron is utilized in mitochondria for heme synthesis via FECH and ABCB6 or transported to the nucleus by PIR, which suppresses autophagy by limiting the cytosolic translocation of HMGB1. Excess iron can lead to oxidative stress and ROS production via the Fenton reaction, which can be countered by NQO1, HMOX1, or G6PD. ACSL4 is a key regulator of ferroptosis, producing PUFAs for subsequent ALOX-mediated lipid peroxidation. MGST1 inhibits ALOX activity through direct interaction, while MUFAs may competitively inhibit PUFA lipid peroxidation. Multiple mechanisms inhibit excessive lipid peroxidation, with GPX4 playing a central role by using GSH to neutralize lipid peroxides. GSH levels are positively regulated by the system xc^- -mediated cystine uptake and subsequent cysteine production, with SLC7A11 and SLC3A2 as key components. GCL (composed of GCLC and GCLM) is the rate-limiting enzyme in GSH synthesis, and GSSG can be reduced back to GSH by GSR. AIFM2 provides a GPX4-independent anti-ferroptotic pathway through various mechanisms, including the production of CoQ10 or vitamin K, or activation of ESCRT-III membrane repair pathways. 4-HNE, an end product of lipid peroxidation, is detoxified by the ALDH family. NFE2L2-dependent genes are highlighted in green, while positive regulators of ferroptosis are shown in red. NFE2L2: NFE2-like basic leucine zipper transcription factor 2; TFRC: transferrin receptor; SLC40A1: solute carrier family 40 member 1; FTH1: ferritin heavy chain 1; FTL: ferritin light chain; MT1G: metallothionein 1G; NCOA4: nuclear receptor coactivator 4; HERC2: HECT and RLD domain containing E3 ubiquitin protein ligase 2; HMOX1: heme oxygenase 1; FECH: ferrochelatase; ABCB6: ATP-binding cassette subfamily B member 6; PIR: pirin; HMGB1: high mobility group box 1; ROS: reactive oxygen species; NQO1: quinone oxidoreductase-1; G6PD: glucose-6-phosphate dehydrogenase; ACSL4: acyl-CoA synthetase long-chain family member 4; PUFAs: polyunsaturated fatty acids; ALOX: lipoxygenase; MGST1: microsomal glutathione S-transferase 1; MUFAs: monounsaturated fatty acids; GPX4: glutathione peroxidase 4; GSH: glutathione; GCL: glutamate-cysteine ligase; GCLC: glutamate-cysteine ligase catalytic subunit; GCLM: glutamate-cysteine ligase

modifier subunit; GSSG: oxidized glutathione; GSR: glutathione reductase; AIFM2: apoptosis-inducing factor mitochondria-associated 2; CoQ10: coenzyme Q10; ESCRT-III: endosomal sorting complex required for transport-III; 4-HNE: 4-hydroxynonenal; ALDH: aldehyde dehydrogenase.

The most studied intrinsic pathway involves GPX4, an enzyme that reduces lipid hydroperoxides to their corresponding alcohols using GSH as a substrate^[27]. GPX4 activity is regulated by selenium^[28], and targeting GPX4 through covalent inhibitors, proteolysis-targeting chimeras, or cell-type-specific degraders can induce ferroptosis to suppress tumor growth^[29]. Moreover, multiple GPX4-independent antioxidant enzymes, such as apoptosis-inducing factor mitochondria-associated 2 (AIFM2, also known as FSP1)^[30,31] and dihydroorotate dehydrogenase (DHODH)^[32], inhibit ferroptosis in cancer cells with low GPX4 expression. However, the role of AIFM2 or DHODH inhibitors in inducing ferroptosis through coenzyme Q10 (CoQ10) interference remains debated^[33]. In certain contexts, AIFM2 inhibits ferroptosis by promoting the endosomal sorting complex required for transport-III (ESCRT-III)-mediated plasma membrane repair^[34] or by contributing to vitamin K production^[35-37], rather than solely affecting CoQ10.

The final stage of ferroptotic cell death is characterized by membrane rupture, although the precise mechanisms remain under investigation, including the possible involvement of pore-forming proteins^[38]. It is generally accepted that excessive lipid peroxidation of polyunsaturated fatty acids (PUFAs), key components of cellular membranes, may directly lead to membrane rupture. The accumulation of toxic lipid metabolites may trigger a positive feedback loop, amplifying ferroptotic cell death and potentially spreading the damage to neighboring cells^[39].

Most ferroptosis inhibitors, such as ferrostatin-1 and liproxstatin-1, act by inhibiting the production of lipid ROS, thereby preventing lipid peroxidation. Acyl-CoA synthetase long-chain family member 4 (ACSL4) is a critical enzyme in the regulation of ferroptosis, as it facilitates the synthesis of PUFA-CoA esters^[40-43], which are highly susceptible to peroxidation by enzymes of the lipoxygenase (ALOX) family or cytochrome P450 oxidoreductase (POR)^[44,45]. ACSL4 mediates at least two downstream pathways, leading to the formation of different PUFA-CoA esters. One pathway involves lysophosphatidylcholine acyltransferase 3 (LPCAT3), which incorporates PUFAs into phosphatidylethanolamines (PEs), making them prime targets for peroxidation^[40,43]. The other pathway involves sterol O-acyltransferase 1 (SOAT1, also known as ACAT1), which produces PUFA-cholesteryl esters (CEs) instead of PUFA-PEs^[46]. Both pathways contribute to lipid peroxidation, with the specific substrates used varying based on the cellular context.

In human pancreatic cancer cells lacking the lipid flippase solute carrier family 47 member 1 (SLC47A1), ACSL4-driven PUFA-CE production is particularly significant in promoting ferroptosis^[46]. Conversely, elevated levels of monounsaturated fatty acids (MUFAs) inhibit ferroptosis, while saturated fatty acids may exert dual roles depending on the cellular environment^[47,48]. For instance, the upregulation of LPCAT1 leads to the incorporation of more saturated fatty acids into membrane phospholipids, increasing phospholipid saturation and reducing PUFA content, thereby inhibiting ferroptosis^[49].

Lipid droplets - organelles that store neutral lipids such as triglycerides and cholesteryl esters - serve as a protective mechanism against ferroptosis by sequestering PUFAs or releasing MUFAs^[50]. However, their degradation through lipophagy promotes ferroptosis by releasing PUFAs back into the cellular environment, where they become susceptible to peroxidation^[51]. This organelle-level regulation of lipid metabolism presents a therapeutic target for modulating ferroptosis sensitivity in cancer cells. Given that lipid droplets contain both PUFAs and MUFAs, further investigation is needed to understand the signaling and mechanisms underlying the selective release of these fatty acids during ferroptosis.

Overall, the interaction between iron and lipid metabolism plays a complex role in regulating ferroptosis sensitivity. Understanding and modulating this pathway holds significant promise for developing new therapies for various diseases, particularly cancer.

INVOLVEMENT OF AUTOPHAGY IN NFE2L2 ACTIVATION OR MODULATION

Autophagy is a lysosome-dependent degradation system that is essential for maintaining cellular homeostasis, particularly under stress conditions^[52,53]. It facilitates the clearance of specific proteins and organelles, thereby playing a crucial role in determining cellular survival or death^[54,55]. Generally, increased non-selective autophagy promotes cell survival by degrading damaged cellular components and recycling nutrients under stress conditions. However, selective autophagy, which targets specific cellular components, can either promote or inhibit cell death depending on the nature of the cargo being degraded. For instance, selective autophagy processes, such as ferritinophagy and lipophagy, promote ferroptosis by modulating iron and lipid metabolism, respectively^[25,51]. In addition to these mechanisms, autophagy also plays a critical role in regulating NFE2L2 activation during ferroptosis through sequestosome 1 (SQSTM1, best known as p62) as first reported in HCC cells following treatment with erastin or sorafenib^[6].

SQSTM1 is a selective autophagy receptor that binds to ubiquitinated proteins and organelles, facilitating their degradation via the autophagy-lysosome pathway. SQSTM1 contains a ubiquitin-associated (UBA) domain that binds polyubiquitinated proteins, tagging them for autophagic degradation. Additionally, SQSTM1 interacts with microtubule-associated protein 1 light chain 3 (MAP1LC3, also known as LC3) through its LC3-interacting region (LIR). This interaction is critical for the sequestration of ubiquitinated cargo into autophagosomes, which are subsequently degraded upon fusion with lysosomes. For example, in HCC and HEK293T cells, SQSTM1 acts as a scaffold to promote the interaction between nuclear receptor coactivator 4 (NCOA4) and MAP1LC3, thereby facilitating ferritin degradation and enhancing sensitivity to ferroptosis^[56]. This process is negatively regulated by the deubiquitinating enzyme ubiquitin-specific peptidase 8 (USP8)^[56].

Autophagy deficiency leads to the accumulation of SQSTM1, which plays a role in positively regulating the transcription factor NFE2L2. Under normal conditions, NFE2L2 is continuously ubiquitinated by the KEAP1-CUL3-ring-box 1 (RBX1) E3 ubiquitin ligase complex, leading to its proteasomal degradation [Figure 3A]. However, when autophagy is impaired, accumulated SQSTM1 interacts with KEAP1 via its KEAP1-interacting region, effectively sequestering KEAP1 away from NFE2L2^[57] [Figure 3B]. This sequestration prevents the ubiquitination and degradation of NFE2L2, leading to its stabilization^[57]. Indeed, RNAi-mediated knockdown of *SQSTM1* increases KEAP1 protein levels, enhances the interaction between KEAP1 and NFE2L2, and promotes the degradation of NFE2L2 in HCC cells in response to ferroptosis inducers, such as erastin and sorafenib^[6]. Furthermore, the knockdown of *KEAP1* reverses the enhanced degradation of NFE2L2 observed following *SQSTM1* depletion in HCC cells after erastin treatment^[6]. These findings demonstrate the critical role of the interaction between SQSTM1 and KEAP1 in maintaining NFE2L2 expression levels during ferroptosis.

Moreover, SQSTM1 undergoes phosphorylation at Ser349 in humans (Ser351 in mice), which enhances its binding affinity for KEAP1, further promoting the sequestration of KEAP1 and resulting in the activation of NFE2L2^[58]. The activation of NFE2L2 under these conditions upregulates the expression of antioxidant response genes, protecting cells from oxidative stress and mitigating ferroptotic damage across various disease models^[6,59-62]. As a feedback mechanism, the release of SQSTM1 during ferroptosis can increase advanced glycosylation end-product specific receptor (AGER, also known as RAGE)-dependent ACSL4 expression, leading to PUFA production for autophagosome formation and subsequent ferroptosis, as observed in pancreatitis models^[63].

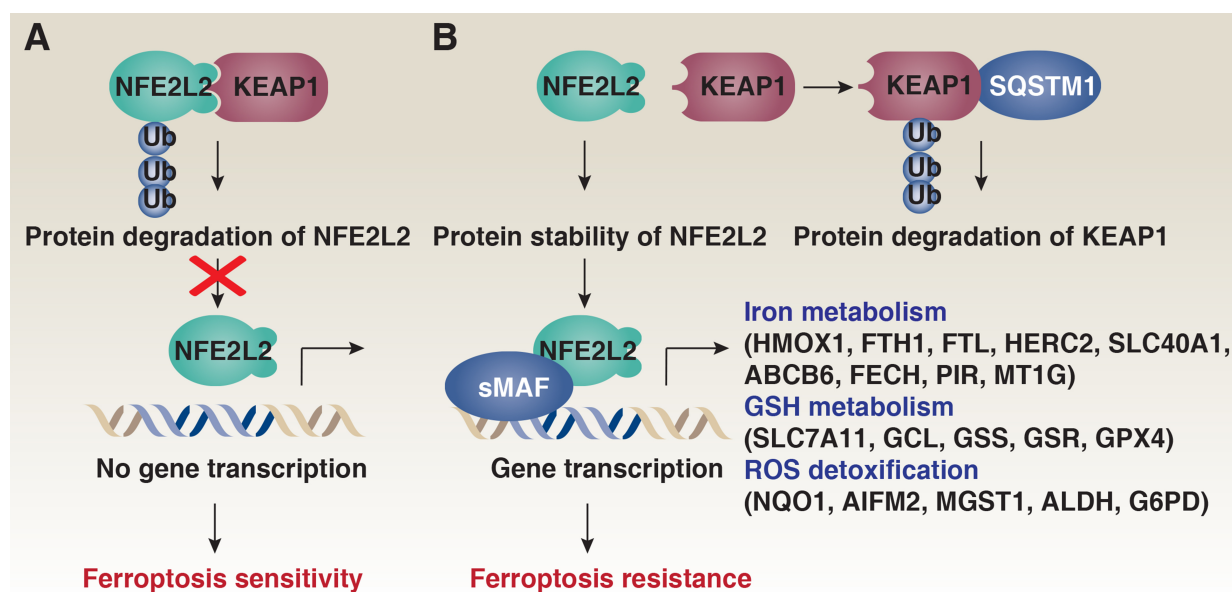


Figure 3. Effects of autophagy impairment on NFE2L2 activation in ferroptosis. (A) Under physiological conditions, NFE2L2 is continuously ubiquitinated by the KEAP1-CUL3 E3 ubiquitin ligase complex, resulting in its proteasomal degradation; (B) When autophagy is impaired, accumulated SQSTM1 interacts with KEAP1, leading to the proteasomal degradation of KEAP1. This interaction sequesters KEAP1 away from NFE2L2, preventing the ubiquitination and degradation of NFE2L2. Consequently, NFE2L2 is stabilized and translocates to the nucleus, where it dimerizes with sMAF proteins. This complex binds to AREs to mediate the transcription of genes involved in iron metabolism, GSH metabolism, and ROS detoxification. NFE2L2: NFE2-like basic leucine zipper transcription factor 2; KEAP1: Kelch-like ECH-associated protein 1; CUL3: cullin 3; SQSTM1: sequestosome 1; sMAF: small musculoaponeurotic fibrosarcoma; AREs: antioxidant response elements; GSH: glutathione; ROS: reactive oxygen species.

In addition to the well-characterized KEAP1-CUL3-RBX1 complex, other E3 ligase complexes, such as the beta-transducin repeat containing E3 ubiquitin protein ligase (BTRC, also known as β -TrCP1)-S-phase kinase-associated protein 1 (SKP1)-CUL1-RBX1 complex and synoviolin 1 (SYVN1, also known as HRD1), are identified as mediators of NFE2L2 degradation during oxidative stress^[64,65]. Although SYVN1 mediates erastin-induced ferroptosis in ovarian cancer cells by facilitating ubiquitination-dependent degradation of SLC7A11^[66], direct evidence for SYVN1-dependent NFE2L2 degradation during ferroptosis remains limited. It is essential to further investigate the specific signals that selectively activate these different E3 ligase complexes in the context of ferroptosis.

NFE2L2-TARGETED GENES IN FERROPTOSIS AND DRUG RESISTANCE

The activation of NFE2L2 is pivotal in protecting cells from ferroptotic death. To achieve this protective function, NFE2L2 regulates a broad spectrum of genes involved in multiple cellular pathways, including those that modulate ROS, detoxify harmful agents, and repair damaged proteins [Figure 2 and Table 1]. Below, we summarize key NFE2L2-targeted genes that regulate ferroptosis inducer-mediated anticancer activity in a context-dependent manner.

Quinone oxidoreductase-1

Quinone oxidoreductase-1 (NQO1) is an enzyme in cellular defense against oxidative stress, primarily functioning within the body's detoxification system. NQO1 catalyzes the two-electron reduction of quinones to hydroquinones, a process that mitigates the formation of ROS and detoxifies quinone-based drugs or those metabolized into quinone structures. NQO1 is frequently upregulated in various cancers, where its overexpression contributes to the development of drug resistance^[67]. Elevated NQO1 levels in tumors correlate with poor prognosis, as the enzyme can reduce the efficacy of chemotherapy. Moreover, NQO1 upregulation has been proposed as a marker of aggressive tumor behavior.

Table 1. NFE2L2 target genes involved in the regulation of ferroptosis in cancer cells

Name	General function	Role in ferroptosis	Tumor type	Ref.
NQO1	Detoxification of quinones	Anti-ferroptotic by reducing ROS and protecting against lipid peroxidation	HCC, lung, pancreatic, and other cancers	[6]
HMOX1	Degradation of heme to biliverdin, iron, and CO	Dual role: promotes ferroptosis through free iron release, but can protect through antioxidant effects	Multiple cancers	[6,74-76]
FTH1	Iron storage	Anti-ferroptotic by sequestering free iron and preventing ROS formation	Multiple cancers	[6]
FTL	Iron storage	Works with FTH1 to prevent ferroptosis by reducing free iron levels	Multiple cancers	[6]
HERC2	E3 ubiquitin ligase regulating NCOA4 degradation	Anti-ferroptotic by inhibiting ferritinophagy, reducing iron availability	HCC	[81]
SLC40A1	Iron exporter	Anti-ferroptotic by reducing intracellular iron levels	Breast, neuroblastoma	[82,83]
ABCB6	Transport of porphyrins and detoxification	Inhibits ferroptosis by protecting cells from oxidative damage	Multiple myeloma	[88]
FECH	Heme synthesis	Inhibition leads to ferroptosis through reduced heme availability	Lung cancer	[89]
PIR	Oxidative stress response	Anti-ferroptotic by reducing DNA damage and inhibiting HMGB1 release	Pancreatic cancer	[92]
MT1G	Metal ion homeostasis and detoxification	Anti-ferroptotic by preventing lipid peroxidation	HCC, renal cell carcinoma	[94]
SLC7A11	Cystine/glutamate antiporter	Anti-ferroptotic by promoting GSH synthesis and ROS neutralization	Ovarian, esophageal, lung	[100,102,103]
GCL	Glutamate-cysteine ligase, GSH synthesis	Anti-ferroptotic by increasing GSH levels	Multiple cancers	[107]
GSS	GSH synthetase, GSH synthesis	Anti-ferroptotic by increasing GSH synthesis	Multiple cancers	[107]
GSR	GSH reductase, GSH regeneration	Anti-ferroptotic by regenerating reduced GSH	Multiple cancers	[107]
GPX4	Reduces lipid peroxides	Anti-ferroptotic by preventing lipid peroxidation	Multiple cancers	[113]
AIFM2	NAD(P)H oxidoreductase	Anti-ferroptotic by reducing CoQ10 and maintaining plasma membrane integrity	Lung, pancreatic, HCC	[30,31]
MGST1	Microsomal GSH S-transferase	Anti-ferroptotic by detoxifying lipid peroxidation products	Pancreatic, gastric, uterine	[116]
ALDH1A1	Detoxification of aldehydes	Anti-ferroptotic by detoxifying lipid peroxidation products	Breast, lung, cervical	[123]
ALDH3A1	Detoxification of aldehydes	Anti-ferroptotic by detoxifying lipid peroxidation products	Glioblastoma, colorectal	[123]
G6PD	NADPH production in the pentose phosphate pathway	Anti-ferroptotic by providing NADPH for GSH regeneration	Renal cell carcinoma, HCC	[129]

NFE2L2: NFE2-like basic leucine zipper transcription factor 2; NQO1: quinone oxidoreductase-1; ROS: reactive oxygen species; HCC: hepatocellular carcinoma; HMOX1: heme oxygenase 1; FTH1: ferritin heavy chain 1; FTL: ferritin light chain; HERC2: HECT and RLD domain containing E3 ubiquitin protein ligase 2; NCOA4: nuclear receptor coactivator 4; SLC40A1: solute carrier family 40 member 1; ABCB6: ATP binding cassette subfamily B member 6; FECH: ferrochelatase; PIR: pirin; HMGB1: high mobility group box 1; MT1G: metallothionein 1G; SLC7A11: solute carrier family 7 member 11; GSH: glutathione; GCL: glutamate-cysteine ligase; GSS: glutathione synthetase; GSR: glutathione reductase; GPX4: glutathione peroxidase 4; AIFM2: apoptosis-inducing factor mitochondria-associated 2; CoQ10: coenzyme Q10; MGST1: microsomal GSH S-transferase 1; G6PD: glucose-6-phosphate dehydrogenase; NADPH: nicotinamide adenine dinucleotide phosphate.

In the context of ferroptosis, NFE2L2-mediated upregulation of NQO1 is implicated in promoting resistance to ferroptosis inducers, such as erastin and sorafenib, in HCC cells^[6]. Conversely, genetic knockdown of *NQO1* enhances the sensitivity of HCC cells to these agents, underscoring its role in modulating ferroptosis susceptibility^[6]. Inhibitors targeting NQO1 are being explored as potential therapeutic strategies to counteract its protective effects in cancer cells, thereby increasing their vulnerability to ferroptosis inducers.

Further complexity in NQO1's role is highlighted by its interaction with various compounds. For instance, tanshinone acts as a coenzyme for NQO1, accepting electrons from flavin adenine dinucleotide to generate reduced tanshinone at the expense of nicotinamide adenine dinucleotide. This reduced form detoxifies lipid peroxyl radicals, thereby inhibiting erastin- or RSL3-induced ferroptosis in HT1080 cells^[68]. In contrast, NQO1 is a predictive target for plumbagin using the PharmMapper database and molecular docking studies^[69]. The growth-inhibitory effects of plumbagin are modulated by NQO1; suppression (via dicumarol) or elimination (via *NQO1* knockdown) of NQO1 reduces plumbagin-induced ferroptosis in glioma cells^[69]. This reduction is attributed to plumbagin enhancing the interaction between NQO1 and tumor protein P53 (TP53, also known as p53), protecting TP53 from degradation and consequently reducing the expression of SLC7A11^[69]. This pro-ferroptotic mechanism of NQO1 is independent of its antioxidant function. Moreover, the deletion of *NQO1* does not affect sensitivity to RSL3 alone^[31]. However, cells lacking both *AIFM2* and *NQO1* exhibit increased sensitivity compared to *AIFM2* knockout U-2 OS cancer cells^[31].

Thus, NQO1 regulates ferroptosis sensitivity through multiple mechanisms involving both its enzymatic and non-enzymatic functions.

Heme oxygenase 1

Heme oxygenase 1 (HMOX1, also known as HO-1), an NFE2L2-dependent inducible enzyme, plays a crucial role in the degradation of heme, a pro-oxidant, into three biologically active products: biliverdin, free iron, and carbon monoxide^[70]. Each of these products has distinct biological activities that contribute to cellular protection. Biliverdin is rapidly converted to bilirubin, a potent antioxidant that scavenges ROS, thereby protecting cells from oxidative damage. Free iron released from heme is sequestered by ferritin, reducing the availability of free iron that could otherwise catalyze the formation of highly reactive hydroxyl radicals via the Fenton reaction. Carbon monoxide, with its anti-inflammatory and vasodilatory properties, further contributes to cellular protection against oxidative stress.

Through its antioxidant functions, particularly via bilirubin and carbon monoxide, HMOX1 mitigates oxidative stress and provides a protective advantage to cancer cells, shielding them from ferroptosis. For instance, in HCC cells, the knockdown of *HMOX1* via shRNA enhances the anticancer efficacy of erastin and sorafenib^[6]. Preclinical studies have shown that inhibition of HMOX1 by zinc protoporphyrin or tin protoporphyrin enhances the efficacy of chemotherapy (e.g., cisplatin)^[71], photodynamic therapy^[72], and radiotherapy^[73]. By inhibiting HMOX1, the protective effects on tumor cells are diminished, increasing their susceptibility to treatment-induced oxidative stress and subsequent cell death.

In some contexts, the free iron released by HMOX1 can enhance sensitivity to ferroptosis. For example, HMOX1 accelerates ferroptotic cell death induced by erastin, BAY 11-7085 (an IκBα inhibitor), or withaferin A in fibrosarcoma, breast cancer, and neuroblastoma cells, respectively^[74-76]. Additionally, EF24, a synthetic analog of curcumin developed as an antitumor compound, induces ferroptosis in osteosarcoma cells by upregulating HMOX1, which in turn inhibits GPX4^[77]. Co-treatment with donafenib and GSK-J4 also induces ferroptosis in HCC cells through the upregulation of HMOX1 expression^[78].

The dual role of HMOX1 in ferroptosis underscores the need for careful examination of the production of biliverdin, free iron, and carbon monoxide, as well as the buffering capacity of ferritin on free iron. Understanding these dynamics is essential for leveraging HMOX1 as a therapeutic target in cancer treatment.

Ferritin heavy chain 1, ferritin light chain, HECT and RLD domain containing E3 ubiquitin protein ligase 2

Ferritin is a spherical protein complex composed of 24 subunits, which include two types: the heavy chain (FTH1) and the light chain (FTL). These subunits function cooperatively to store iron in a non-toxic, soluble form, thereby preventing the catalysis of harmful free radicals by free iron. Ferritin is thus crucial for maintaining iron homeostasis and protecting cells from iron-induced ferroptosis.

FTH1 was first identified as a target gene of NFE2L2 when it was observed that basal *Fth1* mRNA levels in *Nfe2l2*-deficient mice were significantly lower than those in wild-type mice^[79]. The relationship between NFE2L2 activation and FTL was further elucidated when *Ftl* transcript levels were found to be twofold higher in WT compared to *Nfe2l2*-deficient mouse intestines^[80]. In HCC cells, NFE2L2-mediated upregulation of *FTH1* enhances the cellular antioxidant defense by increasing the capacity for iron storage, thereby reducing erastin- or sorafenib-induced ferroptosis^[6]. Conversely, ferritinophagy-mediated degradation of ferritin through autophagy receptor NCOA4 increases ferroptosis sensitivity, which suppresses the growth of various tumor cell types^[25]. The NFE2L2-targeted gene HECT and RLD domain containing E3 ubiquitin protein ligase 2 (*HERC2*) encodes an E3 ubiquitin ligase that facilitates the proteasomal degradation of NCOA4, thereby inhibiting ferritinophagy-dependent ferroptosis^[81]. Further insights into the regulatory mechanisms controlling ferritin induction and degradation may lead to the development of new ferroptosis-based antitumor strategies.

Solute carrier family 40 member 1

Solute carrier family 40 member 1 (SLC40A1), also known as ferroportin-1, is a key iron-exporting protein responsible for transporting iron from the intracellular environment to the extracellular space. It is predominantly expressed in cells involved in iron storage and transport, including enterocytes, macrophages, hepatocytes, and placental cells. This iron-export function is critical for maintaining proper iron homeostasis across various tissues and within the bloodstream.

SLC40A1 is a target gene of NFE2L2, with a putative ARE identified approximately -7 kb upstream of the *SLC40A1* core promoter. NFE2L2-mediated upregulation of *SLC40A1* is anticipated to reduce intracellular iron levels and inhibit ferroptosis. For instance, overexpression of *SLC40A1* inhibits, while knockdown of *SLC40A1* enhances, siramesine and lapatinib-induced ferroptosis by modulating iron efflux in breast cancer cells^[82]. Knockdown of *SLC40A1* also accelerates erastin-induced ferroptosis in neuroblastoma cells^[83], highlighting its role in modulating ferroptotic sensitivity. However, contradictory evidence exists; for instance, other studies have suggested that NFE2L2 induces cisplatin resistance by suppressing the iron export gene *SLC40A1* in ovarian cancer cells^[84], indicating the presence of unknown feedback mechanisms. Nonetheless, the degradation of SLC40A1 through SQSTM1-dependent autophagy or the ring finger protein 217 (RNF217)-dependent ubiquitin-proteasome system enhances sensitivity to erastin- or RSL3-induced ferroptosis in certain cell types, including macrophages and HT1080 cells^[85,86].

In addition, the relationship between hepcidin and SLC40A1 forms a crucial feedback loop that maintains systemic iron homeostasis and modulates ferroptosis sensitivity^[83]. Hepcidin negatively regulates SLC40A1 by promoting its degradation, thereby reducing iron export and controlling circulating iron levels. This balance is essential for preventing both iron deficiency and iron overload.

ABCB6 and FECH ATP binding cassette subfamily B member 6, and ferrochelatase

Heme is a vital molecule consisting of a porphyrin ring complexed with ferrous iron, essential for various biological processes such as oxygen transport and electron transfer. Its synthesis occurs through a complex biochemical pathway involving multiple enzymatic steps and substrates. Disruptions or deficiencies in any of

the enzymes or substrates required for heme synthesis can lead to the accumulation of intermediate compounds, resulting in a group of disorders known as porphyrias^[87]. These disorders are characterized by the buildup of these intermediates in the blood, tissues, and urine, causing a range of clinical symptoms.

Two critical enzymes in this pathway, ATP binding cassette subfamily B member 6 (ABCB6) and ferrochelatase (FECH), are regulated by the transcription factor NFE2L2 and play significant roles in inhibiting ferroptosis when upregulated. ABCB6 acts as a broad-spectrum transporter of porphyrins, enabling the export and import of heme and its precursors across plasma and mitochondrial membranes. It also functions as a detoxifying agent by acting as a heavy metal efflux pump. Recent studies have identified nitidine chloride, a compound discovered through the screening of a natural product library, as a direct inhibitor of ABCB6^[88]. This inhibition promotes ferroptosis and suppresses tumor growth in multiple myeloma by blocking the PI3K-AKT signaling pathway^[88].

FECH, another key enzyme in the heme biosynthesis pathway, catalyzes the insertion of ferrous iron into protoporphyrin IX to form heme B, a crucial step occurring within the matrix-facing side of the inner mitochondrial membrane. Interestingly, the flavonoid chalcone 4,4'-dimethoxychalcone induces ferroptosis in lung cancer cells by upregulating HMOX1 and inhibiting FECH^[89]. This finding suggests that additional transcription factors may work alongside NFE2L2 to regulate the expression and activity of these enzymes, highlighting the complex regulation of heme synthesis and its impact on cell survival and death mechanisms.

Pirin

Pirin (PIR) is a nuclear protein of the cupin superfamily, defined by a conserved β -barrel fold. It plays a crucial role in transcription regulation and oxidative stress response. As a metalloprotein with a non-heme iron binding site, PIR relies on this iron, which alternates between Fe^{2+} and Fe^{3+} , to maintain its structural integrity and participate in redox reactions^[90].

In human pancreatic cancer cell lines, the expression of PIR is upregulated by NFE2L2 in response to ferroptosis-inducing agents (erastin or RSL3)^[91]. Silencing PIR through knockdown experiments increases the levels of γ -H2AX, a marker of DNA damage, and 8-oxo-7,8-dihydro-2'-deoxyguanosine, an indicator of oxidative DNA damage, following treatment with these agents. This increased DNA damage is linked to increased cytoplasmic translocation and extracellular release of high mobility group box 1 (HMGB1) in PIR-deficient cancer cells^[91]. Given that cytosolic HMGB1 can interact with Beclin 1 to activate autophagy-dependent ferroptosis^[92], the upregulation of PIR by NFE2L2 likely serves to retain HMGB1 in the nucleus, thereby inhibiting ferroptosis^[91].

Additionally, cancer cells with a pronounced epithelial-mesenchymal transition (EMT) phenotype - a process where polarized epithelial cells acquire mesenchymal characteristics - are more susceptible to ferroptosis^[93]. Given PIR's role in promoting EMT, investigating whether PIR-mediated EMT influences ferroptosis could offer valuable insights into the interplay between EMT and ferroptosis in cancer progression.

Metallothionein 1G

Metallothionein 1G (MT1G) is a member of the metallothionein family, which comprises small, cysteine-rich proteins characterized by their ability to bind heavy metals through the thiol groups of cysteine residues. These metallothioneins play a critical role in maintaining metal ion homeostasis, protecting cells from metal toxicity, and facilitating the detoxification of harmful substances.

The expression of MT1G during ferroptosis is regulated by the NFE2L2 signaling pathway. In the context of cancer, particularly HCC, the upregulation of MT1G has been linked to resistance against sorafenib, a kinase inhibitor commonly used in cancer therapy^[94]. The regulation of MT1G by sorafenib appears to be independent of its kinase inhibition activity. Instead, overexpression of *MT1G* reduces sorafenib-induced lipid peroxidation and subsequently inhibits ferroptosis. This protective effect of MT1G on cancer cells contributes to the development of drug resistance. Experimental evidence suggests that silencing or inhibiting MT1G expression can enhance the anticancer efficacy of sorafenib by promoting ferroptosis, both *in vitro* and *in vivo*^[94]. This enhancement is particularly significant in clear cell renal cell carcinoma cell lines, such as 769-P and CAKI-1, where MT1G appears to modulate ferroptosis by regulating GSH consumption - a key antioxidant mechanism within these cells^[95].

Clinically, elevated levels of MT1G are associated with poor prognosis in patients undergoing treatment with sorafenib or immune checkpoint inhibitors for various cancers, including liver, colorectal, and prostate cancers^[96-98]. These findings suggest that MT1G not only plays a role in mediating resistance to ferroptosis, but also serves as a potential biomarker for predicting patient outcomes and the effectiveness of ferroptosis-targeted therapies in cancer treatment. Consequently, targeting MT1G could represent a novel therapeutic strategy to overcome resistance and improve the efficacy of current anticancer treatments.

SLC7A11

The heterodimeric amino acid transport system x_c^- is composed of two subunits: the light chain subunit SLC7A11 and the heavy chain subunit SLC3A2. SLC7A11 specifically mediates the transport of extracellular cystine into the cell in exchange for intracellular glutamate, an essential function for maintaining the intracellular pool of cysteine. Cysteine, once imported, is rapidly reduced to cysteine, a key precursor in the synthesis of GSH. GSH is a critical intracellular antioxidant that protects cells from oxidative stress by neutralizing ROS and serves as a necessary cofactor for GPX4.

The regulation of SLC7A11 is intricately linked to the cellular response to oxidative stress and is tightly controlled by various signaling pathways, reflecting the context-dependent nature of its role in ferroptosis. TP53, a well-known tumor suppressor, negatively regulates SLC7A11, thereby sensitizing cells to ferroptosis by reducing cystine uptake and subsequent GSH synthesis^[99]. This regulation by TP53 is particularly significant in the context of cancer, where the loss of TP53 function can lead to enhanced SLC7A11 activity, promoting tumor survival under oxidative stress conditions. Conversely, NFE2L2 and activating transcription factor 4 (ATF4) are positive regulators of SLC7A11^[100,101].

In esophageal squamous cell carcinoma, the upregulation of SLC7A11 by NFE2L2 is particularly prominent. NFE2L2 directly binds to the promoter region of SLC7A11, leading to increased expression of this transporter, which in turn contributes to the resistance to ferroptosis and to radiotherapy^[100]. The therapeutic potential of targeting this pathway is highlighted by studies on brusatol, an inhibitor of NFE2L2. Brusatol induces ferroptosis in bladder cancer cells by suppressing NFE2L2-mediated upregulation of SLC7A11, thereby reducing the cell's antioxidant capacity^[102]. The expression of SLC7A11 is also upregulated in ovarian cancer tissues, playing a critical role in promoting resistance to ferroptosis by enhancing cystine uptake and supporting GSH biosynthesis^[103]. Overall, modulating the NFE2L2-SLC7A11 axis could be a viable strategy for overcoming resistance to ferroptosis in certain cancers.

Moreover, the transcriptional regulation of *SLC7A11* is further influenced by NFE2L2-binding cofactors, such as alternate reading frame (ARF), which is a key mediator of TP53 activation in response to oncogenic signals^[104], and YEATS domain containing 4 (YEATS4, also known as GAS41), a member of the YEATS family of chromatin-associated proteins^[105]. These cofactors enhance the transcriptional activity of NFE2L2,

thereby amplifying the expression of SLC7A11 and contributing to the inhibition of ferroptosis in cancer cells^[104,105]. This amplification of SLC7A11 expression through cofactor interaction underscores the complex regulatory network that governs the cellular response to oxidative stress and ferroptosis.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), one of the most frequently mutated tumor suppressors in cancer, also plays a critical role in modulating SLC7A11 expression. The loss of *PTEN* function, commonly observed in various cancers, leads to increased expression of SLC7A11^[106]. This occurs through the prevention of BTRC-mediated degradation of NFE2L2, which in turn sustains high levels of SLC7A11, thereby reducing the susceptibility of cancer cells to ferroptosis^[106]. The elevated SLC7A11 expression associated with PTEN loss is thought to confer a survival advantage to tumor cells by enhancing their antioxidant defenses, thereby promoting tumorigenesis and resistance to therapy.

glutamate-cysteine ligase, GSH synthetase, and GSH-disulfide reductase

The initial and rate-determining step in GSH synthesis is facilitated by glutamate-cysteine ligase (GCL, formerly termed γ -glutamylcysteine synthetase). GCL is a heterodimeric enzyme composed of two subunits: the catalytic subunit (GCLC) and the modifier subunit (GCLM), which are encoded by distinct genes. GCLC mediates the formation of a γ -carboxyl linkage between glutamate and cysteine, a reaction that necessitates ATP and Mg^{++} as cofactors. GCLM enhances the catalytic efficiency and turnover number of GCLC, lowers the Michaelis constant for glutamate and ATP, and increases the inhibition constant for GSH-mediated feedback inhibition of GCL. The subsequent enzyme, GSH synthetase (GSS), in the GSH biosynthetic pathway catalyzes the conjugation of gamma-glutamylcysteine with glycine to generate GSH. GSH-disulfide reductase (GSR) then catalyzes the reduction of oxidized GSH (GSSG) back to its reduced form (GSH), a pivotal molecule in counteracting oxidative stress and preserving the cell's redox balance.

Importantly, GCLC, GCLM, GSS, and GSR are transcriptional targets of NFE2L2. Their upregulation is implicated in conferring resistance to ferroptosis across various contexts, including chemotherapy and radiation therapy^[107]. While the GSH pathway and its involvement in ferroptosis have garnered increasing attention, GSH also serves as an inhibitor of other forms of regulated cell death, such as apoptosis and cuproptosis. Further elucidating the specific roles of GSH-dependent enzymes and identifying their targets are critical for differentiating the diverse roles of GSH in cell death processes.

GPX4

GPX4 stands out within the GPX family due to its unique ability to reduce lipid hydroperoxides directly within cell membranes, thereby safeguarding cells from lipid peroxidation and ferroptosis. Elevated GPX4 expression is linked to resistance against chemotherapy, highlighting its role in drug resistance. Conversely, pharmacological inhibition of GPX4 can induce ferroptosis, offering a potential strategy to overcome therapy resistance. Impaired protein degradation pathways, including the ubiquitin-proteasome system and autophagy, significantly contribute to the upregulation of GPX4. Moreover, GPX4 expression is regulated at the transcriptional level, and it has been identified as a potential target gene of NFE2L2. Several enzymes involved in protecting against ROS, including GPX4, are upregulated in wild-type mice but not in *Nfe2l2* knockout mice treated with chemopreventive agents, such as 3H-1,2-dithiole-3-thione, isothiocyanate PEITC, and sulforaphane^[80,108,109].

Inhibition of NFE2L2 by trigonelline reverses resistance to GPX4 inhibitor-induced ferroptosis in head and neck cancer cells^[110], implicating NFE2L2 in the development of resistance to GPX4 inhibitors. This resistance is linked to the induction of sestrin 2 (SESN2) expression under endoplasmic reticulum stress and subsequent degradation of KEAP1 via SQSTM1, establishing a connection between endoplasmic reticulum stress, NFE2L2 activity, and ferroptosis resistance^[110]. *SESN2* itself is an NFE2L2-dependent gene, with its

expression induced by NFE2L2 activators such as tert-butylhydroquinone and sulforaphane in hepatocytes^[111], suggesting a positive feedback loop between SESN2 expression and NFE2L2 activation^[112].

In breast cancer cells, NFE2L2-mediated transcriptional activation of GPX4 contributes to radioresistance^[113]. Interestingly, tubastatin A, a selective HDAC6 inhibitor, overcomes both ferroptosis resistance and radioresistance in cancer cells by inhibiting GPX4 enzymatic activity, independent of histone deacetylase 6 (HDAC6) inhibition^[113]. Further research is needed to elucidate the precise mechanisms by which tubastatin A inhibits GPX4 activity and to compare its effects with known GPX4 inhibitors such as RSL3.

Overall, GPX4 is a key driver of ferroptosis resistance; however, its expression also influences other forms of non-ferroptotic cell death, such as pyroptosis. The molecular mechanisms underlying various cell death pathways induced by GPX4 deficiency require further investigation.

AIFM2

AIFM2, a flavoprotein, functions as an NAD(P)H-dependent oxidoreductase with significant roles beyond its initial hypothesized function. Originally, it was believed that AIFM2 might induce apoptosis within mitochondria due to its structural similarity to apoptosis-inducing factor mitochondria-associated 1 (AIFM1). However, recent research has revealed that AIFM2 plays a critical role in a GPX4-independent ferroptosis defense mechanism, particularly in *GPX4*-knockout cells^[30,31]. This defense mechanism is localized to the plasma membrane, where AIFM2 is myristoylated, facilitating its membrane localization and enabling it to function effectively as an oxidoreductase that reduces CoQ10.

CoQ10 is a fat-soluble molecule ubiquitous in human cells, where it is essential for energy production through its involvement in the mitochondrial electron transport chain. Moreover, CoQ10 serves as a potent antioxidant, neutralizing free radicals and thereby protecting cellular membranes from oxidative damage. This antioxidant function of CoQ10 is crucial in the context of ferroptosis. In addition to its role in CoQ10 reduction, increased expression of AIFM2 has been associated with the activation of the ESCRT-III repair machinery^[34]. This activation is crucial for maintaining membrane integrity by mitigating oxidative damage. Furthermore, AIFM2 is linked to the production of reduced vitamin K, another important factor in protecting cellular membranes from oxidative stress^[35,37].

There is a positive correlation between AIFM2 expression and the sensitivity of cancer cells to ferroptosis inducers such as RSL3, ML162, ML210, and erastin. This relationship suggests that targeting AIFM2 could be a promising strategy to overcome ferroptosis resistance in cancer therapy, particularly in tumors that are resistant to conventional treatments. Upregulation of AIFM2 has also been implicated in the activation of the NFE2L2 pathway^[114]. Two AREs are identified in the promoter regions of the *AIFM2* gene, indicating that AIFM2 expression is tightly regulated by NFE2L2. Targeting NFE2L2-dependent AIFM2 expression has shown potential in enhancing ferroptosis-mediated tumor suppression, especially in lung cancer cells deficient in *KEAP1*^[114].

However, further research is necessary to fully understand the context-dependent roles of NFE2L2-mediated AIFM2 expression under various ferroptotic stresses. For example, a recent study demonstrated that AIFM2 confers ferroptosis resistance in *KEAP1*-mutant non-small cell lung carcinoma through both NFE2L2-dependent and independent mechanisms^[115]. Moreover, it would be beneficial to explore the mechanisms by which *GPX4* depletion triggers the AIFM2-mediated anti-ferroptotic pathway, contributing to cell survival. Understanding these pathways could provide new therapeutic targets for cancer treatment and potentially improve the efficacy of ferroptosis-based therapies.

Microsomal GSH S-transferase 1

Microsomal GSH S-transferase 1 (MGST1) is a membrane-bound enzyme primarily localized in the mitochondria, endoplasmic reticulum, plasma membrane, and peroxisomes. MGST1 exhibits dual functions, acting both as a GSH S-transferase (GST) and as a GPX, enabling it to metabolize a wide range of substrates. These substrates include products of lipid peroxidation, halogenated hydrocarbons, and carcinogenic drugs, highlighting its role in cellular detoxification processes.

Beyond its detoxifying capabilities, MGST1 plays a critical role in inhibiting apoptosis. Inducible MGST1 expression, mediated by the activation of the NFE2L2 pathway, inhibits ferroptosis in pancreatic cancer cells^[116]. However, mutations in the ARE of the *MGST1* gene can impair its upregulation during ferroptosis, weakening this protective effect. Functionally, the loss of MGST1, similar to the loss of NFE2L2, reduces the resistance of pancreatic cancer cells to ferroptosis, making them more vulnerable to this form of cell death. Conversely, re-expression of MGST1 in *NFE2L2*-knockdown pancreatic ductal adenocarcinoma cells can restore their resistance to ferroptosis, underscoring the critical role of MGST1 in ferroptosis regulation. Mechanistically, MGST1 has been shown to interact with arachidonate 5-lipoxygenase (ALOX5), inhibiting ALOX5-mediated lipid peroxidation, a key driver of ferroptosis^[116].

The implications of MGST1 extend beyond pancreatic cancer. Elevated MGST1 expression is associated with poor prognosis and ferroptosis resistance in other cancers, including gastric cancer and uterine corpus endometrial carcinoma^[117,118]. In gastric cancer cells, *MGST1* overexpression leads to the inhibition of autophagy, a process that can trigger autophagy-dependent ferroptosis^[119]. Specifically, MGST1 represses the expression of autophagy-related 16-like 1 (ATG16L1) and the conversion of MAP1LC3-I to MAP1LC3-II, both critical steps in autophagy, thereby providing an additional mechanism by which MGST1 confers ferroptosis resistance^[119].

In addition to NFE2L2, the transcription factor zinc finger protein 384 (ZNF384) has also been implicated in the upregulation of MGST1^[120]. In glioma cells, ZNF384-mediated MGST1 expression contributes to resistance against the chemotherapeutic agent temozolomide by negatively regulating ferroptosis^[120]. This highlights the potential of MGST1 as a target for overcoming chemoresistance in glioma.

Collectively, these studies suggest that targeting MGST1, either through NFE2L2-dependent or NFE2L2-independent pathways, represents a promising strategy to enhance ferroptosis-mediated tumor suppression. This approach could potentially improve the efficacy of cancer therapies, particularly in cancers that exhibit resistance to ferroptosis.

ALDH1A1 and ALDH3A1

The aldehyde dehydrogenase 1 (ALDH1) family, a group of isoenzymes within the acetaldehyde dehydrogenase superfamily, contributes to cellular detoxification processes. These enzymes are primarily responsible for the oxidation of acetaldehyde, a toxic byproduct of alcohol metabolism, into acetic acid via alcohol dehydrogenase. This conversion is crucial for maintaining cellular homeostasis and preventing the accumulation of harmful aldehydes that can lead to cellular damage and disease.

Among the ALDH1 family members, ALDH1B1 has garnered particular interest due to its interactions with eukaryotic translation initiation factor 4E (EIF4E) within membrane compartments, especially mitochondria-associated membranes^[121]. This interaction inhibits the ALDH1B1-mediated clearance of 4-hydroxynonenal (4-HNE), a lipid peroxidation byproduct and a potent mediator of ferroptosis. By limiting the detoxification of 4-HNE, the EIF4E-ALDH1B1 complex increases cellular susceptibility to ferroptosis^[121].

The ALDH1 family comprises six functional genes, each with distinct biological roles. Among these, ALDH1A1 and ALDH3A1 have been identified as direct targets of the NFE2L2 pathway. The upregulation of these genes is associated with drug resistance in various cancers. For example, *ALDH1A1* overexpression is linked to resistance against several chemotherapeutic agents, including cyclophosphamide, doxorubicin, and paclitaxel in breast cancer^[122]; paclitaxel in lung cancer^[123]; and cisplatin in cervical cancer^[124]. This resistance mechanism is partly due to the ability of ALDH1A1 to detoxify aldehydes generated by chemotherapy, thereby protecting cancer cells from cytotoxicity. In colorectal cancer, long-term exposure to increasing concentrations of 5-fluorouracil induces a switch from ALDH1A1 to the ALDH1A3 isoform^[125]. This isoform switch is associated with cross-resistance to multiple chemotherapeutic agents, including 5-fluorouracil, oxaliplatin, cisplatin, and irinotecan, making treatment more challenging and reducing the efficacy of conventional therapies^[125].

Therapeutically, targeting NFE2L2-dependent expression of ALDH1A1 and ALDH1A3 offers a promising strategy to enhance the sensitivity of cancer cells to chemotherapy. In pancreatic cancer cells, inhibition of these isoenzymes increases the antiproliferative effects of 5-fluorouracil^[126]. Additionally, the inhibition of ALDH1A1 reduces the stemness of human and mouse glioblastoma cells by inducing ferroptosis, suggesting a potential strategy for targeting cancer stem cells and overcoming resistance^[127].

Interestingly, the microbial metabolite indole-3-acetaldehyde has been reported to upregulate ALDH3A1, which catalyzes the conversion of retinal into nicotinamide adenine dinucleotide^[128]. This reaction is crucial for powering the AIFM2-mediated antiapoptosis system, which contributes to the development and progression of colorectal cancer^[128]. Moreover, the upregulation of ALDH3A1 is implicated in the development of temozolomide-resistant glioblastoma cells, primarily due to impaired ferroptosis, further highlighting the role of ALDH3A1 in cancer resistance mechanisms.

Given the diverse roles of the ALDH1 family in mediating drug resistance, particularly through NFE2L2-dependent pathways, targeting these enzymes represents a potent therapeutic approach. By inhibiting ALDH1A1 and ALDH3A1, it may be possible to overcome resistance to chemotherapy and enhance the efficacy of existing cancer treatments, providing new avenues for combating resistant cancer types.

Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme that catalyzes the rate-limiting first step of the pentose phosphate pathway (PPP), an essential metabolic route that operates parallel to glycolysis. The primary function of the PPP is to generate reducing equivalents in the form of nicotinamide adenine dinucleotide phosphate (NADPH) and to produce ribose-5-phosphate, a precursor for nucleotide synthesis. G6PD facilitates the conversion of glucose-6-phosphate into 6-phosphoglucono- δ -lactone, simultaneously reducing NADP⁺ to NADPH. This step is vital as NADPH is a crucial cofactor in various anabolic reactions and is indispensable for maintaining cellular redox balance.

NADPH plays a fundamental role in protecting cells against oxidative stress by serving as a reducing agent in the regeneration of GSH and thioredoxin, two critical antioxidants that neutralize ROS. The ability of NADPH to sustain antioxidant defenses is particularly important in red blood cells (RBCs), which are continuously exposed to high levels of oxygen and lack mitochondria, the primary source of alternative NADPH production. Without sufficient NADPH, RBCs are highly vulnerable to oxidative damage, which can result in hemolysis and various hemolytic disorders, particularly in individuals with G6PD deficiency^[129].

While *G6PD* deficiency is associated with hemolytic anemia, it has also been linked to a lower risk and mortality for certain types of cancer. This observation suggests a complex role of *G6PD* in cancer biology, where its activity may be crucial for the survival and proliferation of cancer cells. Many cancers exhibit upregulated *G6PD* expression, which enhances their ability to cope with oxidative stress, supports rapid cell growth, and contributes to metabolic reprogramming that favors tumor progression.

In cancer cells, the overexpression of *G6PD* and the subsequent activation of the pentose phosphate pathway are often driven by oncogenic signaling pathways, including the NFE2L2 pathway. The upregulation of *G6PD* through NFE2L2 activation not only aids in the detoxification of ROS but also contributes to the resistance of cancer cells to ferroptosis^[130]. This resistance to ferroptosis is particularly evident in clear cell renal cell carcinoma and HCC, where elevated *G6PD* expression and PPP activation have been shown to protect against oxidative damage and promote cancer cell survival. Further understanding the regulation and function of *G6PD* in various cancers may offer new insights into therapeutic strategies aimed at targeting metabolic vulnerabilities and overcoming resistance to ferroptosis, potentially leading to more effective cancer treatments^[131].

TARGETING NFE2L2-ASSOCIATED FERROPTOSIS REGULATION IN CANCER THERAPY

Integrating NFE2L2-associated ferroptosis regulation into cancer therapies is critical for advancing the clinical potential of this research in oncology. NFE2L2 plays a central role in promoting resistance to ferroptosis by upregulating key antioxidant defenses, such as GPX4 and SLC7A11, which neutralize ROS and lipid peroxides. This defense mechanism provides cancer cells with a survival advantage, especially in oxidative and stress-inducing environments, highlighting the therapeutic potential of targeting NFE2L2. Inhibition of NFE2L2 can sensitize cancer cells to ferroptosis-inducing agents, thereby enhancing the efficacy of chemotherapy, radiotherapy, and targeted therapies that rely on oxidative stress to eliminate tumor cells^[132-137]. Furthermore, combining NFE2L2 inhibition with immunotherapies (e.g., CD19-targeted CAR T-cell therapeutics) could amplify tumor immunogenicity by increasing ROS accumulation and ferroptotic cell death, thereby boosting antitumor immune responses^[138]. These strategies, when employed in conjunction with existing treatments such as radiotherapy and targeted drug delivery systems, offer the potential for more comprehensive and durable cancer therapies by addressing both tumor survival mechanisms and NFE2L2-driven resistance pathways^[139,140].

Despite the progress in understanding NFE2L2-regulated genes involved in ferroptosis, there remains a gap in the systematic investigation of each gene's specific contribution to ferroptosis regulation. Among these genes, GPX4 is of particular interest due to its critical role in reducing lipid peroxides and promoting ferroptosis resistance. Targeting the GPX4 pathway holds significant therapeutic potential, particularly when combined with standard treatments such as chemotherapy and radiotherapy, which are less effective in apoptosis-resistant tumors. However, current GPX4 inhibitors face challenges such as poor metabolic stability, limited bioavailability, and side effects *in vivo*^[141,142]. Recent advancements in pharmacological approaches to modulate GPX4 activity and expression, such as covalent inhibitors (e.g., LOC1886), proteolysis targeting chimera degraders (e.g., dGPX4), and cell-type-specific degraders (e.g., N6F11), offer promising strategies for inducing ferroptosis as a therapeutic intervention in cancer treatment^[29]. These novel approaches offer the potential to overcome the limitations of existing inhibitors, enhancing both efficacy and safety in clinical applications. Expanding research in this area is critical for optimizing ferroptosis modulation and may pave the way for innovative therapeutic strategies that utilize NFE2L2 inhibition to overcome drug resistance and improve clinical outcomes across various cancer types.

CONCLUSIONS AND PERSPECTIVES

Ferroptosis is an iron-related form of oxidative stress driven by excessive lipid peroxidation, and its mechanisms and regulation are highly context-dependent^[143]. The activation of the SLC7A11-GSH-GPX4 pathway plays a central role in defending against ferroptosis. In cells with low GPX4 expression, GPX4-independent pathways, such as AIFM2, are more effective in inhibiting ferroptosis. However, conditions where both GPX4-dependent and independent systems are active also occur in cells or tissues with normal GPX4 expression. These phenotypes reinforce the notion that the body possesses multiple antioxidant systems that can function either dependently or independently to combat oxidative stress.

Nonetheless, overactivation of NFE2L2-regulated genes plays a critical role in both GPX4-dependent and independent antioxidant pathways, contributing to key processes such as scavenging ROS, synthesizing GSH, producing NADPH, metabolizing heme and iron, detoxifying xenobiotics, and chelating metals. Moreover, NFE2L2-driven metabolic reprogramming maintains redox homeostasis, further promoting resistance to therapy. Therefore, therapeutic strategies aimed at modulating NFE2L2 activity could offer more effective means of regulating ferroptosis and overcoming drug resistance than current approaches. A deeper understanding of the interplay between NFE2L2 signaling, ferroptosis regulation, and resistance mechanisms is essential for the development of novel cancer therapies.

Despite the exciting advances in understanding NFE2L2 and ferroptosis, several challenges and questions remain to be addressed^[142]. First, there is a lack of comprehensive studies examining the dynamics of NFE2L2-target gene induction during ferroptosis. This is crucial because some target genes act as early-response regulators, while others function as late-response regulators. Second, based on current literature, the discussed NFE2L2-regulated genes also modulate other forms of oxidative cell death. It remains unclear how these target genes selectively regulate different cell death pathways. Third, excessive production of reduced antioxidants can also trigger cell death, a phenomenon we previously termed “reductive cell death”. It is necessary to evaluate the threshold at which antioxidants switch from anti-death to pro-death functions. Finally, the current NFE2L2 inhibitors and activators generally affect the overall function of NFE2L2. There is a need for collaboration between industry and academia to design next-generation NFE2L2-related compounds that can selectively modulate NFE2L2 expression or function in specific tissues or cells, or at the level of individual NFE2L2-targeted genes. This specificity is crucial for reducing side effects.

DECLARATIONS

Authors' contributions

Conceptualized the framework of this review and wrote the paper: Tang D, Kang R

Availability of data and materials

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Competing endogenous RNAs (ceRNAs) and drug resistance to cancer therapy

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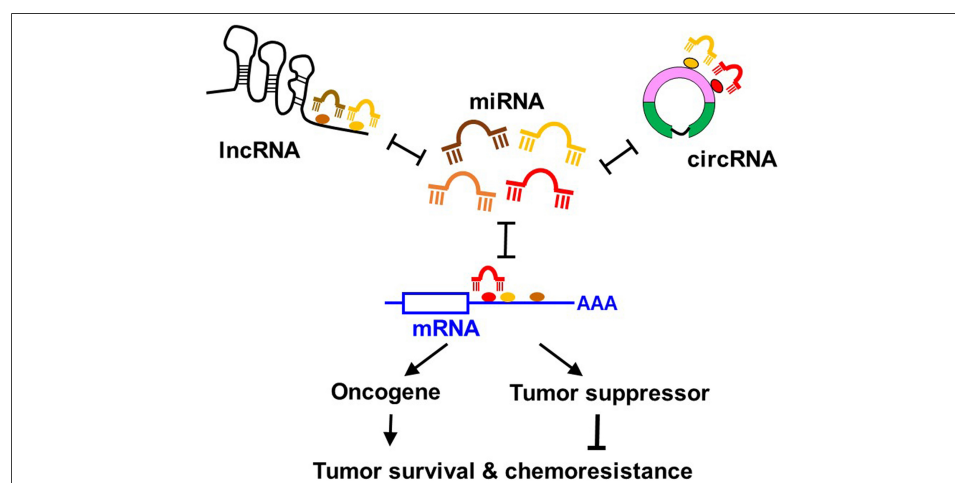
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Abstract

Competing endogenous RNAs (ceRNAs) are transcripts that possess highly similar microRNA response elements (MREs). microRNAs (miRNAs) are short, endogenous, single-stranded non-coding RNAs (ncRNAs) that can repress gene expression by binding to MREs on the 3' untranslated regions (UTRs) of the target mRNA transcripts to suppress gene expression by promoting mRNA degradation and/or inhibiting protein translation. mRNA transcripts, circular RNAs (circRNAs), long non-coding RNAs (lncRNAs), and transcribed pseudogenes could share similar MREs, and they can compete for the same pool of miRNAs. These ceRNAs may affect the level of one another by competing for their shared miRNAs. This interplay between different RNAs constitutes a ceRNA network, which regulates many important biological processes. Cancer drug resistance is a major factor leading to treatment failure in patients receiving chemotherapy. It can be acquired through genetic, epigenetic, and various tumor microenvironment mechanisms. The involvement of ceRNA crosstalk and its disruption in chemotherapy resistance is attracting attention in the cancer research community. This review presents an updated summary of the latest research on ceRNA dysregulation causing drug resistance across different cancer types and chemotherapeutic drug classes. Interestingly, accumulating evidence suggests



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that ceRNAs may be used as prognostic biomarkers to predict clinical response to cancer chemotherapy. Nevertheless, detailed experimental investigations of the putative ceRNA networks generated by computational algorithms are needed to support their translation for therapeutic and prognostic applications.

INTRODUCTION

microRNAs (miRNAs) are short (~22 nucleotides in length), endogenous, single-stranded non-coding RNAs (ncRNAs) that repress gene expression at the post-transcriptional level in eukaryotic organisms. They bind to miRNA response elements (MREs) on the 3' untranslated regions (UTRs) of the target mRNA transcripts with imperfect complementarity, subsequently suppressing gene expression by either promoting mRNA degradation or inhibiting protein translation^[1]. Recent research demonstrated that mRNA transcripts, circular RNAs (circRNAs), long non-coding RNAs (lncRNAs), and transcribed pseudogenes could share highly similar MREs, and they interact with the same set of miRNAs to regulate miRNA activity. These competing endogenous RNAs (ceRNAs) work as decoys for a particular miRNA, thereby abrogating the interaction of the miRNA to its target mRNA^[2-5]. The expression of ceRNA usually exhibits a negative correlation with the miRNAs that carry the relevant recognition sequences^[6]. On the other hand, the expression levels of a pair of ceRNAs are usually positively correlated^[7]. Figure 1 depicts a schematic diagram illustrating the ceRNA networks of mRNA/miRNA/ncRNA to induce cancer drug resistance. Numerous *in silico* algorithms have been established to predict putative pairs of ceRNA in accordance with their shared miRNAs and cellular expression^[8].

The ceRNA regulatory network involving different RNA molecules can have significant implications for various biological processes and disease progression. This area of active research is providing insights into the complexity of gene regulation. While most ceRNA interactions reported in the literature reflect only single binding partners (i.e., one miRNA and a pair of ceRNA transcripts), emerging evidence suggests that ceRNA crosstalk occurs in an interconnected manner as a network. Besides direct interactions via shared miRNAs, indirect interactions may also mediate a significant effect on ceRNA modulation. Further investigation of the ceRNA crosstalk should also involve analysis of potential miRNAs and ceRNA networks. Targeting nonessential nodes within the regulatory network will not produce a useful therapeutic response because cancer cells could overcome the damage through alternative survival pathways. Instead, the critical juncture of the ceRNA network may represent potential therapeutic targets for cancer therapy.

Cancer drug resistance is a major unresolved obstacle to successful cancer chemotherapy. It is a multifactorial phenomenon that is mediated by increased drug efflux, reduced susceptibility to apoptosis, accelerated DNA damage repair, aberrant drug biotransformation, mutations in cellular molecular targets, and reduced anticancer immunity^[9]. Accumulating evidence suggests that dysregulated ceRNA regulation is involved in the drug resistance mechanisms affecting numerous anticancer drugs.

In this review, we present the latest investigation about how ceRNA dysregulation triggers drug resistance to cancer therapy across different cancer types and chemotherapeutic drug classes. Representative preclinical studies and clinical findings are highlighted. Emerging prognostic biomarkers based on ceRNAs to predict clinical response to cancer chemotherapy will also be discussed.

SPECIFIC CERNAL NETWORK MEDIATING DIFFERENT CHEMORESISTANCE MECHANISMS

By sequestering miRNAs and preventing them from interacting with their target mRNAs, ceRNAs are known to indirectly modulate the expression of genes regulating drug efflux, drug biotransformation, apoptosis, DNA repair, and other mechanisms associated with chemoresistance. Liu *et al.* reported the first

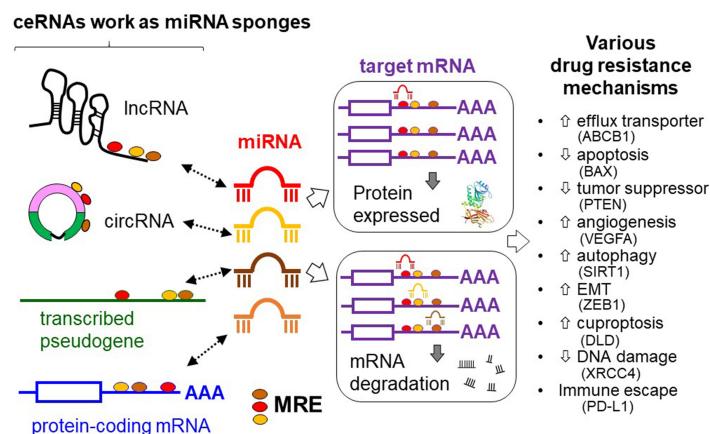


Figure 1. An overview of the dysregulation of ceRNA regulatory network to induce different drug resistance mechanisms (including efflux transporter overexpression, reduced apoptosis, downregulated tumor suppressor, increased angiogenesis, induced autophagy, increased EMT, increased cuproptosis, reduced DNA damage, and immune escape). Representative downstream effectors leading to the different resistance mechanisms are listed in parentheses. RNA molecules could communicate with one another through miRNA and MRE. The expression of one RNA could influence the level and activity of another RNA by sequestering their common miRNA(s). ceRNA: Completing endogenous RNA; EMT: epithelial-mesenchymal transition; miRNA: microRNA; MRE: microRNA response element; circRNA: circular RNA; lncRNA: long non-coding RNA.

comprehensive analysis of ceRNAs in drug resistance across different cancer types and anticancer drug classes^[10]. In their study, using information about the shared miRNAs and correlation of lncRNA and mRNA expressions retrieved from public databases (lncRNA expression data from TANRIC; mRNA expression signature of cancer cell lines from CCLE; regulatory relationship between miRNA-lncRNA and miRNA-gene according to DIANA-LncBase and miRTarBase), a general ceRNA network was first constructed from 183 lncRNAs and 379 mRNAs. Distinct ceRNA modules (encompassing 138 drugs and 19 cancer types) related to cancer drug resistance were identified in 758 drug-cancer conditions, where there are significantly more differentially-expressed lncRNA and mRNA between drug-resistant and -sensitive cancer cell lines^[10]. Importantly, the functional analysis indicated that resistance-related biological processes (including accelerated cell proliferation, enhanced DNA damage repair, and reduced apoptosis) were enriched in these drug resistance-related ceRNA modules. It is noteworthy that some anticancer drugs (such as the multitargeted kinase inhibitors dasatinib and sunitinib) share the same modules^[10]. These drugs may exhibit similar drug resistance mechanism(s). Moreover, a Jaccard index (a measure of the similarity of different ceRNA modules) of all clinically approved drugs has been computed to examine the possibility of multidrug resistance (MDR). Anticancer drugs with a high Jaccard index were found to possess similar structural backbones^[10]. It is logical because drugs with a high chemical similarity will likely display a similar spectrum of therapeutic efficacy and are more likely to be affected by MDR. The information may help clinicians identify a better treatment choice for drug-refractory tumors.

ceRNA dysregulation induces resistance to traditional cancer chemotherapy

Upregulation of MDR efflux transporters

Overexpression of the MDR efflux transporters in cancer cells is a major mechanism causing chemoresistance. To et al. published a comprehensive review recently about the regulation of MDR transporters by ncRNAs, which provided the latest update about the significant impact of ncRNAs on chemoresistance in cancer therapy and novel approaches for its circumvention^[11]. ncRNAs (including miRNA, lncRNA, and circRNA) are important regulators of cancer cell proliferation, apoptosis, and metabolism^[12,13]. lncRNAs refer to non-coding transcripts longer than 200 nucleotides in length, which are not translated into proteins^[14,15]. They are involved in the regulation of chromatin structure and transcription, splicing, various forms of RNA processing, editing, localization and stability, and protein

Table 1. Representative ceRNA networks promoting overexpression of multidrug resistance ABC efflux transporters in chemoresistant cancer cells

Efflux transporter	Dysregulated ncRNA	Anticancer drug	Cancer type	Ref.
ABCB1	DANCR (lncRNA; upregulated)	Docetaxel	PCa	[129]
	FENDRR (lncRNA; downregulated)	Doxorubicin	CML	[130]
	FTHIP3 (lncRNA; upregulated)	Paclitaxel	BC	[131]
	GAS5 (lncRNA; downregulated)	Doxorubicin	BC	[34]
	HOTAIR (lncRNA; upregulated)	-	HCC	[132]
	LINC00355 (lncRNA; upregulated)	Cisplatin	Bladder cancer	[133]
	LUCAT1 (lncRNA; upregulated)	Mitoxantrone	OS	[134]
	ROR (lncRNA; upregulated)	Cisplatin	OS	[135]
	SNHG16 (lncRNA; upregulated)	-	CRC	[136]
	UCA1 (lncRNA; upregulated)	Imatinib	Leukemia	[27]
	circ_0004674 (circRNA; upregulated)	Doxorubicin	OS	[137]
	CACS15 (lncRNA; upregulated)	Oxaliplatin	CRC	[138]
	circ_0076305 (circRNA; upregulated)	Cisplatin	NSCLC	[139]
ABCC1	KCNQ10T1 (lncRNA; upregulated)	Oxaliplatin	HCC	[140]
	linc00518 (lncRNA; upregulated)	Doxorubicin	BC	[29]
	linc00707 (lncRNA; upregulated)	Cisplatin	NSCLC	[141]
	NR2F1-AS1 (lncRNA; upregulated)	Oxaliplatin	HCC	[142]
ABCC2	circABCC2 (circRNA; upregulated)	-	HCC	[143]
	ANRIL (lncRNA; upregulated)	Cisplatin	Rb	[33]
ABCG2	circSETD3 (circRNA; upregulated)	Gefitinib	NSCLC	[144]
	HOTAIR (lncRNA; upregulated)	Oxaliplatin	GC	[145]

ceRNA: Competing endogenous RNA; ncRNA: non-coding RNA; lncRNA: long noncoding RNA; PCa: prostate cancer; CML: chronic myeloid leukemia; BC: breast cancer; HCC: hepatocellular carcinoma; OS: osteosarcoma; CRC: Colorectal cancer; circRNA: circular RNA; NSCLC: non-small cell lung cancer; Rb: retinoblastoma; GC: gastric cancer.

translation and localization through interactions with RNA, DNA, and protein^[16-18]. Numerous lncRNAs are also known to bind with miRNAs, thereby regulating gene expression of important biological processes^[19,20]. On the other hand, circRNA is a single-stranded ncRNA subfamily in which its 5' and 3' ends join to form a covalently closed continuous loop^[21]. In a typical circRNA molecule, the 3' or 5' ends normally present in an RNA are joined together to form a closed loop, which makes them stable against RNA exonuclease-mediated degradation^[22]. Apart from governing transcription, splicing, translation, and post-translational modifications, circRNAs also regulate gene expression by sequestering miRNAs^[23-25]. To this end, numerous lncRNAs and circRNAs are known to function as molecular decoys to sponge miRNAs and wipe out their interaction with the target mRNAs, subsequently forming ceRNA networks^[26]. Table 1 summarizes the representative lncRNA/circRNA-miRNA-ABC efflux transporter regulating ceRNA machineries that have been shown to promote MDR to various chemotherapeutic drugs in different cancer types. A few representative examples are highlighted below.

In chronic myeloid leukemia (CML) K562 cells, the upregulation of lncRNA UCA1 was shown to increase the MDR transporter ABCB1 expression and induce resistance to the ABCB1 substrate imatinib^[27]. Mechanistically, lncUCA1 sequestered miR-16 and revoked the miRNA-mediated repression on ABCB1 mRNA. Most recently, this lncUCA1-miR-16-5p-ABCB1 regulatory network was also confirmed to regulate ABCB1 expression in human placental BeWo cells by RNA pull-down assay^[28].

In the doxorubicin-resistant breast cancer cell line (MCF-7/ADR), both mRNA and protein expressions of the lncRNA linc00518 and the MDR efflux transporter ABCC1 were found to be significantly upregulated relative to the parental counterpart (MCF-7)^[29]. Consistent with typical ceRNA pairs, genetic silencing of linc00518 was found to inhibit the ABCC1 expression through upregulation of miR-199a. To this end, the plasma level of circulating miR-199a-5p exhibited a positive correlation with the disease progression of breast cancer patients^[30], thus supporting the potential use of linc00518 and miR-199a as novel prognostic biomarkers for drug-refractory breast cancer.

In a glioblastoma tumor xenograft mouse model, the high expression of LINC00479 was found to correlate well with the rapid growth of the tumor^[31]. Detailed mechanistic investigation revealed that LINC00479 sponged miR-134 to increase c-Myc expression, thereby inducing the MDR transporter ABCC1 and triggering temozolomide resistance^[31]. This study established a co-expression relationship between MYC and ABCC1, and that overexpression of MYC could increase ABCC1 levels in glioma cells. Moreover, using rescue experiments, the regulation of ABCC1 by LINC00479 was shown to be mediated by miR-134 whereas the regulation of ABCC1 by miR-134 was mediated by MYC, thus verifying the LINC00479/miR-134/MYC/ABCC1 ceRNA network. In gemcitabine-resistant bladder cancer cells, the antisense lncRNA (FOXD1-ASP1) was found to express at a very high level, which sequestered miR-143 to upregulate the MDR transporter ABCC3 expression^[32].

It has been reported that the expression of a few chemoresistance-causing lncRNAs was induced by specific transcription/epigenetic factors at the transcription level (i.e., via the lncRNA promoters). In cisplatin-resistant retinoblastoma cells, the lncRNA antisense non-coding RNA in the INK4 locus (ANRIL) was upregulated due to increased binding of the hypoxia-inducible factor (HIF-1 α) to the lncRNA promoter^[33]. The elevated expression of ANRIL subsequently upregulates the MDR efflux transporter ABCG2 by sequestering miR-328^[33].

The ceRNA network involving lncRNA and miRNA was also reported to drive the MDR phenotype in cancer patient tumor specimens. The drug resistance mechanism has been studied recently in breast cancer tissues from a cohort of 10 responders and 16 non-responders to a neoadjuvant chemotherapeutic regimen (epirubicin + cyclophosphamide combination followed by docetaxel monotherapy)^[34]. Compared to the tumor tissues from the responders, those from the non-responders exhibited a remarkably lower expression of the lncRNA growth arrest-specific 5 (GAS5) (reduction by ~60%) but a significantly higher expression of the MDR efflux transporter ABCB1 (by > 2-fold)^[34]. In a cell culture study, a doxorubicin-resistant breast cancer cell model (MCF-7/ADR) was also found to express significantly higher levels of ABCB1 but lower levels of GAS5 than the drug-sensitive MCF-7 parental cells^[34]. Mechanistic investigation revealed that lncRNA GAS5 upregulated Dickkopf-2 (DKK2), thereby sequestering miR-221-3p and inhibiting the Wnt/ β -catenin pathway^[34]. To this end, β -catenin and transcription factor 4 (TCF4) were known to promote ABCB1 mRNA transcription by interacting with the *ABCB1* promoter^[35]. Thus, reduced expression of GAS5 in MDR breast cancer cells could upregulate Wnt/ β -catenin signaling and induce the MDR transporter ABCB1 expression.

Apart from the regulation of MDR transporters by ceRNAs, other key chemoresistance mediators are also induced by the dysregulation of ceRNA networks, as described below. [Table 2](#) summarizes various other cancer drug resistance mechanisms triggered by ceRNA dysregulation, which affect different classes of conventional chemotherapeutic anticancer drugs. Key clinical findings are summarized in [Table 3](#).

Table 2. Representative ceRNA networks promoting the various chemoresistance mediators in preclinical studies

Chemoresistance mediator (effector gene)	Anticancer drug(s)	Dysregulated ncRNA	Cancer type	Ref.
Apoptosis (BAX)	Cisplatin	lncRNA XIST (upregulated)	NSCLC	[39]
Apoptosis (Aurora B kinase)	Cisplatin	lncRNA XIST (upregulated)	GC	[146]
Autophagy (SIRT1)	5-FU	lncRNA H19 (upregulated)	CRC	[61]
Autophagy (STX17, RAB33B, UVRAG)	Paclitaxel	Protein-coding mRNA SLC7A11 (downregulated)	OC	[147]
Cell cycle regulation (CCND1)	Doxorubicin	lncRNA PVT1 (upregulated)	OS	[148]
Cell proliferation (KPNA4)	Paclitaxel	circRNA circ_ZFR (upregulated)	NSCLC	[40]
Cuproptosis (DLD)	Tamoxifen	lncRNA C6orf99 (upregulated)	TNBC	[68]
DNA damage response/G2 cell cycle checkpoint (WEE1)	Temozolomide	lncRNA FOXD3-AS1 (upregulated)	GBM	[149]
EMT	Oxaliplatin	lncRNA H19 (upregulated under hypoxic condition)	CRC	[62]
EMT (ZEB1)	Doxorubicin	lncRNA HULC (upregulated)	HCC	[63]
Glutamine metabolism (GLS)	Cisplatin	lncRNA NEAT1 (upregulated)	MB	[150]
Glycolysis (GOT1)	Cisplatin	circRNA circGOT1 (upregulated)	ESCC	[151]
Mitophagy (p62)	Lenvatinib	lncRNA LINC01607 (upregulated)	HCC	[152]
Senescence (SALL1, METAP1, DCAF11)	Sunitinib	lncRNA LINC00461 (upregulated)	RCC	[153]
Tumor suppression (PTEN)	Cisplatin, paclitaxel, and docetaxel	lncRNA HOTAIR (upregulated)	CC	[38]
Transcription factor critical for the Hippo pathway (TEAD1)	Gemcitabine	lncRNA MKLN1-AS (upregulated)	PC	[154]
Tyrosine kinase (BCR-ABL)	Imatinib	circRNA circCRKL (upregulated)	CML	[155]

ceRNA: Competing endogenous RNA; ncRNA: non-coding RNA; lncRNA: long non-coding RNA; NSCLC: non-small cell lung cancer; GC: gastric cancer; 5-FU: 5-fluorouracil; CRC: colorectal cancer; OC: ovarian cancer; OS: osteosarcoma; TNBC: triple negative breast cancer; GBM: glioblastoma; EMT: epithelial mesenchymal transition; HCC: hepatocellular carcinoma; MB: medulloblastoma; ESCC: esophageal squamous cell carcinoma; RCC: renal cell carcinoma; CC: cervical cancer; PC: pancreatic cancer; CML: chronic myeloid leukemia.

Inhibition of apoptosis

The PI3K/Akt/mTOR signaling pathway plays a central role in regulating cancer cell survival, proliferation, and apoptosis^[36]. PTEN is an important tumor suppressor that inhibits PI3k/Akt/mTOR to promote apoptosis^[37]. In cervical cancer, Zhang *et al.* reported that the lncRNA HOTAIR could sponge miR-29b, which indirectly inhibited PTEN by upregulating SP1 expression^[38]. Interestingly, miR-29b did not affect promoter methylation of PTEN, but it regulated PTEN by targeting the SP1 transcription factor. HOTAIR was shown to induce resistance of cervical cancer cell lines HeLa and SiHa to cisplatin, docetaxel, and paclitaxel, which could be reversed by miR-29b upregulation. In non-small cell lung cancer (NSCLC), the lncRNA XIST was reported to sponge miR-520 and induce cisplatin resistance by regulating the apoptotic gene BAX via the p53 signaling pathway^[39]. Recently, a circRNA circ_ZFR was reported to be highly upregulated in paclitaxel-resistant NSCLC tumor specimens and cell lines^[40]. To this end, circ_ZFR knockdown was shown to overcome paclitaxel resistance by reducing KPNA4 expression and inducing apoptosis via sponging miR-195-5p.

Inhibition of tumor suppressor genes

The loss of function of tumor suppressor genes is well known to cause cancer drug resistance^[41]. Phosphatase and tensin homolog on chromosome 10 (PTEN) is an extensively studied tumor suppressor^[42]. PTEN deficiency and its dysfunction leads to aggressive tumor phenotype and reduced response to anticancer therapy. Tay *et al.* were one of the first research teams to identify endogenous protein-coding transcripts

Table 3. Representative clinical data showing the significance of ceRNA dysregulation in mediating cancer drug resistance

Cancer type	Dysregulated ncRNA	miRNA involved	mRNA target of miRNA	Ref.
BC	lncRNA GAS5 (lower expression in chemotherapy (epirubicin + cyclophosphamide combination)-refractory patients)	miR-221-3p	ABCB1 (efflux transporter-mediated resistance)	[34]
BC	lncRNA C6orf99 (highly expressed in ER+ BC patients resistant to endocrine therapy)	miR-370-3p, miR-432-5p	DLD (cuproptosis)	[67]
BC	lncRNA LINC00589 (can be used as an independent prognostic factor for discriminating trastuzumab responders)	miR-100 and miR-452	DLG5 and PRDM16 (tumor suppressor)	[80]
CML	lncRNA HULC (elevated in CML patients with advanced clinical stages)	miR-200a	c-myc and Bcl-2 (apoptosis)	[85]
CRC	circNCOA3 (overexpressed in patients resistant to anti-PD-1 mAb)	miR-203-3p	CXCL1 (immune checkpoint)	[107]
CRC	Exosomal ciRS-122 (elevated in serum samples of oxaliplatin refractory CRC patients)	miR-122	PKM2 (glycolysis)	[70]
CRC	H19 (increased in recurrent patients)	miR-194-5p	SIRT1 (autophagy)	[61]
ESCC	Exosomal lncRNA PART1 (elevated serum level in patients demonstrating poor response to gefitinib)	miR-129	Bcl-2 (apoptosis)	[87]
GBM	Exosomal lncRNA SBF2-AS1 (elevated in serum sample of temozolomide resistant patients)	miR-151a	XRCC4 (DNA double strand break repair)	[75]
HCC	HULC (highly upregulated in patients with more advanced TNM staging)	miR-200a-3p	ZEB1 (EMT)	[63]
NSCLC	circCPA4 (low level in tumor) associated with better prognosis in patients on PD-L1 immunotherapy	let-7	PD-L1 (immune checkpoint)	[106]

ceRNA: Competing endogenous RNA; ncRNA: non-coding RNA; miRNA: microRNA; BC: breast cancer; lncRNA: long non-coding RNA; ER+: estrogen receptor-positive; CML: Chronic myeloid leukemia; CRC: colorectal cancer; DLD: dihydrolipoamide dehydrogenase; PKM2: pyruvate kinase M2; ESCC: esophageal squamous cell carcinoma; GBM: glioblastoma; HCC: hepatocellular carcinoma; EMT: epithelial mesenchymal transition; NSCLC: non-small cell lung cancer.

(SERINC1, VAPA, CCR4-NOT, and CNOT6L) as PTEN ceRNAs that regulate PTEN expression in a miRNA-dependent manner^[43]. As PTEN and its ceRNAs share the same miRNAs, deletion of individual ceRNA by siRNAs allowed more miRNAs to interact with PTEN 3' untranslated region (3'UTR), thereby reducing PTEN mRNA and protein levels. Moreover, the mutual reciprocal regulation of PTEN and its ceRNAs was demonstrated by the fact that PTEN downregulation modulates its ceRNA expression and PTEN ceRNAs are coexpressed with PTEN in patient specimens^[43]. Pseudogenes are DNA sequences that share high sequence similarity to a known gene, but they are not coded into proteins^[44]. Due to the high homolog between pseudogenes and their protein-coding partners, pseudogenes may sequester the shared miRNAs and prevent them from binding to their authentic protein-coding transcripts. A representative example is PTENP1, the pseudogene of PTEN, which competes with PTEN for the same set of miRNAs through numerous conserved MREs^[45]. The direct association between PTENP1 and PTEN expression in tumor specimens from colon cancer patients suggests that PTENP1 transcript may act as a tumor suppressor by regulating PTEN expression^[45,46]. Intriguingly, PTENP1 is known to produce both sense and antisense transcripts to modulate PTEN expression. The PTENP1 sense transcript shares high sequence similarity with PTEN, thus competing with PTEN for binding specific miRNAs to affect PTEN expression. Meanwhile, two functionally independent isoforms (PTENP1-AS- α and PTENP1-AS- β) are transcribed from the antisense strand. PTEN1-AS- α has been shown to bind to the 5'UTR (promoter) of PTEN, which recruits the epigenetic modifiers (including EZH2 and DNMT3A) to suppress PTEN transcription. On the other hand, PTEN1-AS- β was known to bind to the PTEN1 sense transcript, thus forming a complex that is exported to the cytoplasm. As PTEN1-AS- β lacks a poly-A tail, it stabilizes the PTENP1 sense transcript, which works as a miRNA sponge to post-transcriptionally regulate PTEN.

Recent studies reported an intriguing enrichment of 3'UTR shortening among transcripts that could serve as ceRNAs for tumor suppressor genes^[47]. The 3'UTR of mRNA contains recognition sequences for various regulatory elements, including miRNA binding sites. Interestingly, some mRNA transcripts display differential 3'UTR length. This could be mediated by alternative polyadenylation (APA), alternative splicing, or other mechanisms^[48]. The 3'UTR length could influence the availability of miRNA binding sites and, therefore, disrupt the ceRNA regulatory network^[47,49].

Theoretically, long 3'UTRs tend to harbor more miRNA binding sites and provide more opportunities for miRNA-mediated regulation. It follows that ceRNAs with longer 3'UTRs may have a higher chance to compete for shared miRNAs, thereby sequestering them and affecting the expression of the target genes. This could lead to complex ceRNA networks and regulatory interactions. On the other hand, shorter 3'UTRs usually possess fewer miRNA binding sites, thus limiting ceRNA regulatory potential. This could lead to reduced competition for miRNAs and thus minimize the effect on target gene expression. The relevance of differential mRNA 3'UTR length in ceRNA regulation lies in its effect on modulating the availability of miRNA binding sites and shaping the ceRNA network^[48].

Recent transcriptome-wide studies have revealed that more than 70% of human genes exhibit APA^[50,51]. As a result of APA, numerous genes have been reported to generate multiple mRNA isoforms bearing differential 3'UTR length, where key RNA regulatory elements are found^[52]. Mounting evidence suggests that APA can give rise to differential 3'UTR usage according to tissue type, cell state, and environmental condition, thereby coordinating post-transcriptional regulation of numerous genes driving the carcinogenesis process^[53,54]. To this end, mRNAs harvested from highly proliferative or tumorigenic cells have shorter 3'UTRs, which enables their escape from miRNA-mediated repression of mRNA stability and protein synthesis^[50]. A widespread recurrent tumor-specific APA regulation in multiple cancer types has recently been identified^[55]. The prevailing hypothesis is that 3'UTR shortening could activate proto-oncogenes to promote carcinogenesis through the escape of miRNA-mediated repression. Interestingly, according to a recent study correlating the sensitivity of anticancer drugs with APA events characterized by RNA-seq data from The Cancer Genome Atlas (TCGA) data sets^[56], the top drug class correlated with global 3'UTR shortening are DNA topoisomerase I inhibitors that are commonly used in treating colorectal cancer.

When mRNA transcripts with shortened 3'UTRs lose miRNA recognition sites and no longer sponge the miRNAs, the released miRNAs become available to repress their ceRNA partners [Figure 2]. In various cancer types, the disruption of ceRNA crosstalk by mRNA 3'UTR shortening has been reported for tumor suppressor genes^[47,57,58]. Intriguingly, genetic silencing of the master 3'UTR-shortening regulator NUDT21 was shown to repress a few tumor suppressor genes (including PHF6 and LARP1) in a ceRNA-dependent manner and promote cancer proliferation^[47]. Mechanistically, two miRNAs (miR-3187-3p and miR-549) targeting PHF6 were shown to participate in the ceRNA crosstalk. As depletion of DICER1 could abolish PHF6 and NUDT21 crosstalk, the regulation is miRNA-dependent. Moreover, PHF6 3'UTR-mediated luciferase activity was shown to be rescued by the miR-3187-3p antagomir or NUDT21 siRNA.

Promotion of tumor angiogenesis

In lung cancer, the lncRNA H19 was known to promote tumor angiogenesis by regulating the anti-angiogenic miRNAs^[59]. Interestingly, expression of the lncRNA H19 is often induced by cigarette smoke. This smoking-associated H19 dysregulation was shown to provoke angiogenesis by altering the expression of numerous miRNAs (including miR-29, miR-30a, miR-107, miR-140, miR-148b, miR-199a and miR-200) and lead to chemoresistance by inhibiting BiP, DLL4, HIF1 α , PDGFB, PDGFRA, and VEGFA^[59]. Additionally, H19 was also shown to induce tumor-specific pyruvate kinase M2 (PKM2), which is pivotal for the Warburg effect and tumor angiogenesis^[59].

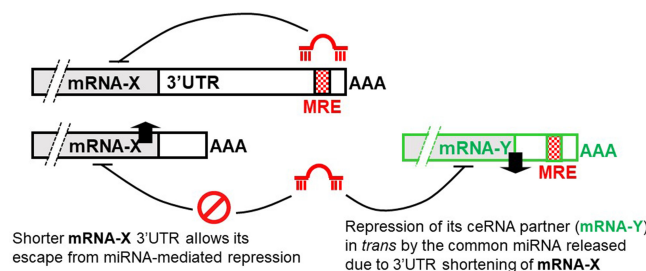


Figure 2. Schematic diagram illustrating how mRNA 3'UTR shortening represses its ceRNA partner *in trans* by releasing the common miRNA(s). 3'UTR shortening of mRNA-X allows its escape from miRNA-mediated repression, therefore increasing mRNA-X expression. However, the release of the common miRNA(s) from binding to the long 3'UTR of mRNA-X will result in the repression of the ceRNA partner mRNA-Y. 3'UTR: 3' untranslated region; ceRNA: completing endogenous RNA; miRNA: microRNA.

Induction of autophagy

Autophagy is a cellular degradation and recycling process that plays a dual role in cancer by functioning as a cell survival or death mechanism^[60]. The dysregulation of ceRNAs has been linked with drug resistance by inducing autophagy. In CRC, the expression of H19 was found to be remarkably increased in recurrent CRC patient tumor samples^[61]. H19 was demonstrated to induce autophagy via SIRT1 to cause 5-fluorouracil (5-FU) resistance. The overexpression of H19 was shown to promote the conversion of LC3-I to LC3-II, thus leading to a remarkable increase in LC3 aggregation and autophagosome formation but downregulation of the autophagy receptor protein p62 in 5-FU-resistant CRC cells. SIRT1 is a direct target of miR-194-5p. Mechanistically, H19 was shown to sponge miR-194-5p, as demonstrated by gene reporter and immunoprecipitation assays, to induce SIRT1 expression in 5-FU-resistant CRC cells^[61].

Induction of epithelial-mesenchymal transition

In CRC, the expression of the lncRNA H19 was induced under hypoxic conditions and in oxaliplatin-resistant cells^[62]. H19 was shown to work as a ceRNA of miR-675-3p to regulate epithelial-mesenchymal transition (EMT) and lead to chemoresistance. Importantly, H19 downregulation could overcome hypoxia-induced chemoresistance by sequestering miR-675-3p to regulate EMT^[62].

In HCC, the lncRNA HULC is highly upregulated, which is associated with advanced TNM staging, metastases, recurrence, and poor drug response^[63]. HULC was shown to sequester miR-200a-3p, which targets and inhibits the transcription factor ZEB1. ZEB1 is critical for EMT and its overexpression is known to drive tumor progression, thus upregulating ZEB1 to mediate EMT and promote metastasis and chemoresistance^[63].

In cervical cancer, HOTAIR was found to promote EMT and induce chemoresistance via a miR-29b/PTEN/PI3K ceRNA network^[38]. HOTAIR was shown to bind with miR-29b. Consistent with the ceRNA mechanism, the expression of HOTAIR and miR-29b is negatively correlated with each other in cervical cancer. While HOTAIR induced cell migration and chemoresistance to cisplatin, docetaxel, and paclitaxel, miR-29b was shown to inhibit EMT. Specifically, miR-29b mimics were shown to downregulate PI3K and enhance cancer drug response.

Recently, a tamoxifen resistance-related ceRNA network has been constructed and validated for breast cancer exhibiting enhanced migration and invasion (EMT phenotype)^[64]. Differentially expressed mRNAs (DEmRNAs) were screened from drug-resistant breast cancer cells by GEO2R. The 20 top-ranked DEmRNAs were associated with 113 upstream miRNAs and 501 lncRNAs. Among these mRNA/miRNA/lncRNA, 7 mRNAs, 22 lncRNAs, and 11 miRNAs were selected to construct a ceRNA

network contributing to tamoxifen resistance in breast cancer. Ultimately, after incorporation of data from GEPIA differential gene expression and Kaplan-Meier survival analyses, 4 mRNAs, 4 lncRNAs, and 3 miRNAs were found to be significantly associated with poor drug response and prognosis^[64]. The differential gene expression was confirmed by quantitative real-time PCR analysis, thus verifying the novel therapeutic targets for tamoxifen resistance in the constructed ceRNA network.

Induction of cuproptosis

Cuproptosis is a new copper metabolism-dependent cell death mechanism^[65,66]. In triple-negative breast cancer, oral administration of the bioavailable copper chelator tetrathiomolybdate has been shown to deplete mitochondrial copper content and reduce cellular energy production, which is significantly correlated with favorable patient survival^[67]. A ceRNA network has been constructed using differentially expressed genes related to cuproptosis in estrogen receptor-positive (ER+) breast cancer^[68]. DLD was found to be a critical cuproptosis-related gene in ER+ breast cancer resistance to endocrine therapy by evaluating the intersection of the protein-protein interaction analysis, differentially expressed genes between the sensitive breast cancer cell lines, and the prognostic cuproptosis-related genes (CRGs)^[68]. The lncRNA C6orf99 was predicted to be a ceRNA that regulates DLD by sponging hsa-miR-370-3p and hsa-miR-432-5p.

Induction of glycolysis

Cancer cells are known to undergo metabolic reprogramming, including increased glycolysis, to promote cell proliferation, metastasis, and chemoresistance^[69]. In CRC, Wang *et al.* reported that the circRNA ciRS-122 was highly upregulated in oxaliplatin-resistant cells^[70]. CiRS-122 was shown to sponge miR-122, thus upregulating human pyruvate kinase M2 (PKM2) to promote glycolysis and drug resistance^[70]. Exosomes are small membrane-bound extracellular vesicles secreted from various cell types. They carry specific nucleic acids, metabolites, and cellular proteins to facilitate intracellular communication^[71]. Numerous ncRNA are secreted in tumor-derived exosomes and transferred to neighboring cancer cells to mediate the chemoresistance phenotype via a ceRNA mechanism^[40,72,73]. Importantly, in oxaliplatin-refractory CRC patients, a high expression level of exosomal ciRS-122 in serum samples was significantly associated with unfavorable clinical response to chemotherapy. Moreover, the delivery of siRNA against ciRS-122 via exosomes was shown to reverse oxaliplatin resistance in cell culture and tumor-bearing mouse models by inhibiting the ciRS-122/miR-122/PKM2 ceRNA network, thus inhibiting glycolysis and drug resistance^[70]. Collectively, drug-resistant cancer cells may exploit ciRS-122 to transfer the chemoresistance phenotype via exosomes to neighboring sensitive cells. Moreover, targeting ciRS-122 may be a novel strategy to overcome oxaliplatin resistance.

Alteration of DNA damage response

In ovarian cancer, the lncRNA urothelial carcinoma associated 1 (UCA1) was reported to mediate cisplatin resistance via transfer in tumor cells-derived exosomes^[74]. Mechanistic investigation indicated that UCA1 worked as a ceRNA of miR-143 to increase the expression of its target FOS-like 2 (FOSL2) mRNA, thereby promoting DNA repair and the drug resistance phenotype. In glioblastoma patients, high serum exosomal lncRNA SBF2-AS1 was shown to contribute to temozolomide resistance^[75]. Mechanistically, SBF2-AS1 was shown to sequester miR-151a to increase the expression of X-ray repair cross-complementing 4 (XRCC4), thus promoting repair of DNA double-strand break and leading to temozolomide resistance^[75].

ceRNA dysregulation induces resistance to targeted cancer therapy

Targeted cancer therapy refers to the approach that specifically inhibits key molecular signaling pathway(s) to elicit the anticancer effect. Numerous small molecule tyrosine kinase inhibitors have been developed as targeted cancer drugs, which have revolutionized the practice of oncology^[76]. However, most cancer patients

receiving targeted cancer therapy inevitably relapse and develop resistance to the treatment. Various mechanisms are known to contribute to targeted drug resistance, including direct target reactivation, aberrant activation of downstream oncogenes, engagement of parallel oncogenic pathways, and other adaptive cancer survival mechanisms^[77]. Emerging evidence suggests that ceRNA crosstalk plays a critical role in drug resistance to many targeted anticancer drugs.

A representative example of ceRNA-mediated drug resistance to targeted chemotherapy is the interaction between the lncRNA H19 and the tumor suppressive miR-200a in breast cancer. The role of the lncRNA H19 in various cancer hallmarks has been recently reviewed by Hashemi *et al.*^[78]. The biological function of H19 in most cancer types is oncogenic, and therefore, high H19 expression is generally correlated with enhanced tumor growth, cell cycle progression, EMT induction, and elevated metastasis. H19 is also well known to trigger chemo- and radio-resistance in cancer cells. Numerous downstream target genes and molecular pathways for lncRNA H19 have been identified and validated, which include miRNAs, RUNX1, STAT3, β -catenin, and FOXM1. To this end, H19-miR-200a constitutes a ceRNA network to mediate resistance to the targeted chemotherapy drug trastuzumab, commonly used for treating HER2-positive breast cancer^[79]. H19 acts as a ceRNA to sequester miR-200a, thus preventing it from binding to its target mRNAs ZEB1 and ZEB2. ZEB1 and ZEB2 are important transcription factors involved in the EMT process, which is associated with increased tumor invasiveness and chemoresistance. The increased expression of ZEB1 and ZEB2 promotes EMT and contributes to drug resistance. Inhibition of H19 or ectopic expression of miR-200a could sensitize trastuzumab-resistant breast cancer cells^[79].

Bai *et al.* recently reported another LINC00589-dominated ceRNA network regulating resistance to HER2-targeted therapy, cancer stemness properties, and multidrug resistance in breast cancer^[80]. The lncRNA LINC00589 was reported as an independent prognostic factor for identifying trastuzumab (HER2 inhibitor) responders. Intriguingly, LINC00589 was found to simultaneously sponge miR-100 and miR-452 to relieve their repression of two tumor suppressors (DLG5 and PRDM16), thereby inhibiting cancer proliferation and counteracting drug resistance^[80]. The two novel ceRNA networks (LINC00589/miR-100/DLG5 and LINC00589/miR-452/PRDM16) may be exploited as useful prognostic markers and novel therapeutic targets for drug-refractory HER2-positive breast cancer.

Sorafenib (a multikinase inhibitor of VEGFR and PDGFR) is the targeted drug of choice for HCC. The let-7 family of miRNAs has been shown to inhibit the expression of the antiapoptotic protein Bcl-xL and potentiate sorafenib-induced apoptosis in HCC^[81]. It has been proposed that the antitumor efficacy of sorafenib could be compromised by high H19 levels by sponging miR-let-7^[82]. Another lncRNA NEAT1 was also reported to promote resistance of HCC to sorafenib by sponging miR-355, thus relieving sorafenib-induced inhibition of the c-Met/Akt pathway^[83]. On the other hand, NEAT1 could also work as a ceRNA of miR-204 to increase the expression of the Autophagy Related 3 (ATG3), subsequently inducing autophagy and leading to sorafenib resistance^[84].

In CML, the lncRNA HULC was shown to promote imatinib resistance by a miR-200a/c-myc/Bcl-2 ceRNA regulatory network^[85]. HULC was shown to be remarkably overexpressed in both leukemia cell lines and hematopoietic cells from CML patients. The elevated HULC level was remarkably associated with more advanced clinical stages of CML. Importantly, HULC silencing was shown to inhibit the activation of PI3K and AKT, and potentiate imatinib-induced apoptosis, which was associated with the downregulation of c-Myc and Bcl-2. Mechanistically, HULC was shown to modulate c-Myc and Bcl-2 by sponging miR-200a^[85].

NcRNAs are also transferred in tumor-derived exosomes to mediate targeted drug resistance via ceRNA-related mechanisms. In NSCLC cells, tumor-derived exosomal circRNA_102481 has been reported to mediate EGFR-TKI resistance by sponging miR-30a-5p to modulate ROR1^[86]. Interestingly, high

expression of circRNA_102481 in exosomes isolated from the peripheral blood of NSCLC patients was positively correlated with more advanced TNM stage, poor tumor differentiation status, prominent brain metastasis, and dismal progression-free survival and overall survival. Genetic silencing of circRNA_102481 or administration of exosomes encapsulated with si-circRNA_102481 could inhibit EGFR-TKI resistance and promote apoptosis. Therefore, exosomal circRNA_102481 may represent a useful diagnostic biomarker and novel therapeutic target for EGFR-TKIs resistance in NSCLC. In esophageal squamous cell carcinoma (ESCC), the lncRNA PART1 was enriched in exosomes and it acted as a ceRNA of miR-129 to upregulate Bcl-2 and promote gefitinib resistance^[87]. Importantly, a high serum level of exosomal PART1 was found to be significantly associated with poor response to gefitinib therapy in ESCC patients. In advanced renal cell carcinoma, exosome-transmitted lncRNA Activated in RCC with Sunitinib Resistance (lncARSR) was shown to mediate sunitinib resistance by sponging miR-34/miR-449 to increase AXL and c-Met expression^[88]. The lncRNA urothelial carcinoma associated 1 (UCA1) was enriched in ovarian cancer-derived exosomes, and it was reported to mediate gefitinib resistance by sequestering miR-143 to increase FOSL2 expression^[89].

ceRNA dysregulation induces resistance to cancer immunotherapy

There is accumulating evidence to suggest the potential impact of ceRNA regulation on cancer immunotherapy response. The regulation of ceRNAs could affect cancer immunotherapy by regulating the immune checkpoint molecules. Jiang *et al.* recently published a comprehensive review of the regulatory role of lncRNAs and circRNAs in the PD-1/PD-L1 pathway and the relevance to the efficacy of immune checkpoint inhibitors^[90]. Programmed cell death protein (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are the two most extensively studied immune checkpoint molecules that are exploited by cancer cells to evade immune surveillance^[91]. Immune checkpoint blockade with anti-CTLA-4 (ipilimumab and tremelimumab), anti-PD-1 (nivolumab and pembrolizumab), or anti-PD-ligand (PD-L1) (durvalumab, atezolizumab, and avelumab) monoclonal antibodies is revolutionizing cancer therapy, which induces durable tumor responses and overall survival benefit in a wide variety of cancer types^[92]. However, some patients do not respond to or develop resistance to immune checkpoint inhibitors, such as anti-PD-1 antibodies. Understanding the mechanisms underlying drug resistance in immunotherapy is crucial for improving patient outcomes.

PD-1 is an inhibitory receptor present on activated immune cells, including T cells, B lymphocytes, natural killer cells, macrophages, dendritic cells, and monocytes, which normally function to blunt both adaptive and innate immune responses. Upon binding of PD-1 to its major ligand PD-L1, which is expressed on tumor cell surface, T cell antitumor immunity will be suppressed. The anti-PD-1/PD-L1 antibodies were designed as immune checkpoint inhibitors for cancer therapy. They work by binding to inhibitory PD-1 receptors on tumor-reactive T cells and PD-L1 on tumor cells, respectively, thereby disrupting the PD-1/PD-L1 interaction and reactivating the antitumor T cell-mediated cell cytotoxicity. Cancer patients with heavy tumor mutational burden, abundant pre-treatment tumor-infiltrating T cells, and elevated pre-treatment PD-L1 levels on tumor cells are expected to respond more favorably to anti-PD-1/PD-L1 immunotherapy^[93].

PD-L1 expression can be regulated by lncRNAs that compete for miRNAs targeting PD-L1 mRNA, thereby promoting immune escape in various cancer types^[94]. Table 4 summarizes representative ceRNA networks modulating PD-1/PD-L1 expression and their effects on cancer immunotherapy. Figure 3 illustrates the induction of cancer immunotherapy resistance due to ceRNA dysregulation-mediated PD-L1 upregulation in different cancer types. In breast cancer, the lncRNA tissue differentiation-inducing non-protein coding RNA (TINCR) was known to upregulate PD-L1 expression both *in vitro* and *in vivo*, thus promoting breast cancer progression. Importantly, TINCR knockdown was shown to significantly potentiate the antitumor efficacy of PD-L1 inhibitors in breast cancer in animal studies. Mechanistically, TINCR was shown to serve a dual function: it sequesters miR-199a-5p and inhibits its transcription, leading to increased PD-L1 expression

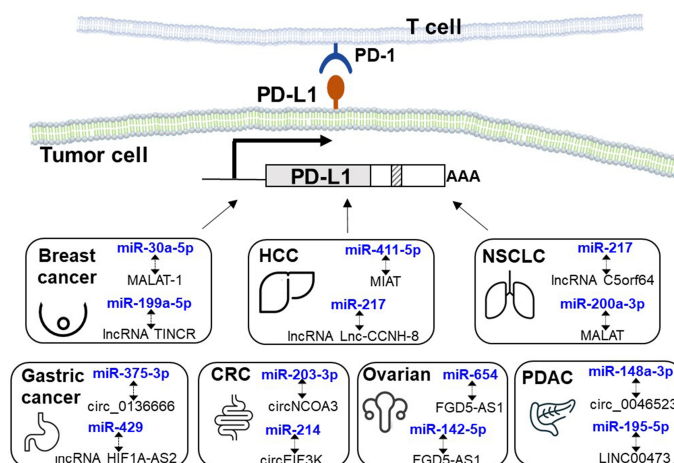


Figure 3. Resistance to cancer immunotherapy due to upregulation of PD-L1 mediated by ceRNA dysregulation in different cancer types. Representative ceRNA networks (miRNA - ncRNA interaction) are shown. PD-L1: Programmed cell death ligand 1; ceRNA: completing endogenous RNA; miRNA: microRNA; ncRNA: non-coding RNA.

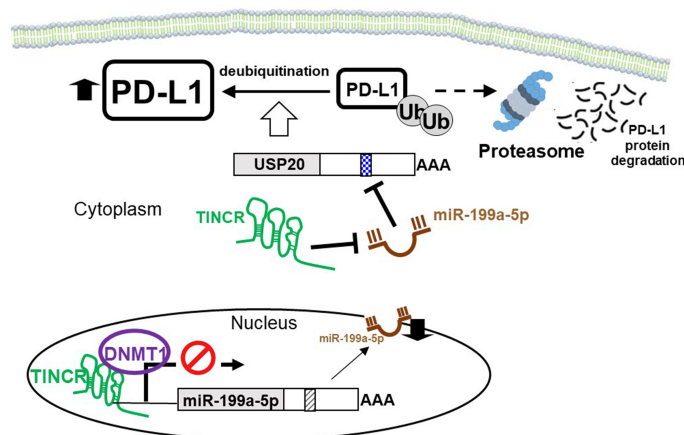


Figure 4. Schematic diagram showing a dual mechanism of PD-L1 regulation by a lncRNA TINCR in breast cancer to mediate resistance to cancer immunotherapy. TINCR was shown to increase PD-L1 expression by upregulating the USP20 via a dual mechanism. TINCR is expressed both in the nucleus and cytoplasm of breast cancer cells. In the cytoplasm, TINCR sponges miR-199a-5p and upregulates USP20 mRNA, thus increasing PD-L1 expression by suppressing its ubiquitination. In the nucleus, TINCR recruits DNMT1 to the gene promoter of miR-199-5p and promotes DNA methylation, thereby inhibiting the transcription of miR-199-5p. PD-L1: Programmed cell death ligand 1; lncRNA: long non-coding RNA; TINCR: tissue differentiation-inducing non-protein coding RNA; USP20: ubiquitin-specific protease 20.

[Figure 4]^[95]. TINCR expression was detected in both the nucleus and cytoplasm of breast cancer cells. In the cytoplasm, TINCR was found to work as a molecular sponge of miR-199a-5p and upregulate the stability of the ubiquitin-specific protease 20 (USP20) mRNA via a ceRNA regulatory machinery, thus increasing PD-L1 expression by suppressing its ubiquitination^[95]. In the nucleus, TINCR was found to recruit DNMT1 to the gene promoter of miR-199a-5p and promote DNA methylation, thereby inhibiting the transcription of miR-199-5p. Therefore, TINCR knockdown may be used as a novel strategy to potentiate PD-L1 immunotherapy in breast cancer. Similarly, CRC tumor cells have been reported to release exosomes enriched in the lncRNA KCNQT1, which regulates the ubiquitination of PD-L1 via a miR-30a-5p/USP22 pathway to facilitate immune evasion^[96]. According to TCGA database, the expressions of KCNQT1 and miR-30a-5p are negatively correlated in tumor tissues. A high level of KCNQT1 in CRC tumors was shown to sequester miR-30a-5p, thereby relieving the repression of the ubiquitin-specific peptidase USP22. It follows that PD-L1 expression is upregulated due to less ubiquitination, subsequently inhibiting CD8⁺ T cell response to induce CRC immune tolerance.

Table 4. Representative ceRNA networks modulating PD-1/PD-L1 expression and their effect on cancer immunotherapy

Cancer type	Dysregulated ncRNA	miRNA involved	mRNA target of miRNA	Ref.
BC	TINCR (lncRNA)	miR-199a-5p	USP20	[95]
CRC	MIR17HG (lncRNA)	miR-375	NF-κB/RELA	[156]
DLBCL	SNHG14 (lncRNA)	miR-5590-3p	ZEB1	[157]
HCC	RNAFOXDI-AS1 (lncRNA)	miR-615-3p	PI3K/AKT	[158]
NSCLC	hsa_circ_0020714 (circRNA)	miR-30a-5p	SOX4	[159]
OC	EMX2OS (lncRNA)	miR-654	AKT3	[160]
PC	PMSB8-AS1 (lncRNA)	miR-382-3p	STAT1	[161]
PCa	lncAMPC (lncRNA)	miR-637	JAK1-STAT3	[162]
Thy	RP11-424C20.2 (pseudogene)	miR-378a-3p	UHRF1	[163]
TNBC	GATA3-AS1 (lncRNA)	miR-676-3p	COP55	[164]

ceRNA: Competing endogenous RNA; ncRNA: non-coding RNA; miRNA: microRNA; BC: breast cancer; CRC: colorectal cancer; DLBCL: diffuse large B cell lymphoma; HCC: hepatocellular carcinoma; NSCLC: non-small cell lung cancer; OC: ovarian cancer; PC: pancreatic cancer; PCa: prostate cancer; Thy: thymomas; TNBC: triple negative breast cancer.

In hepatocellular carcinoma (HCC), the lncRNA Lnc-CCNH-8 is highly expressed and correlates with poor prognosis^[97]. Zhao *et al.* recently reported that Lnc-CCNH-8 could inactivate T cells *in vitro* and suppress antitumor immunity in immunocompetent mice *in vivo*^[97]. Mechanistically, Lnc-CCNH-8 was shown to sequester miR-217 and, therefore, upregulate PD-L1 expression. Moreover, Lnc-CCNH-8 also stabilized PD-L1 via a miR-3173/Phakophilin 3 (PKP3) regulatory machinery. PKP3 is involved in deubiquitination of PD-L1. Therefore, overexpression of Lnc-CCNH-8 and PKP3 could upregulate PD-L1 levels in tumors. In addition, experimental mice bearing tumors with high Lnc-CCNH-8 expression are highly responsive to PD-L1 blockade treatment. Interestingly, HCC patients with high levels of plasma exosomal Lnc-CCNH-8 showed remarkably higher treatment responses to immune checkpoint inhibitors. Collectively, Lnc-CCNH-8 forms a novel ceRNA network to induce immune escape from CD8⁺ T cell-mediated cancer-killing effect by upregulating PD-L1 in a miR-217/miR-3173-dependent manner^[97]. Critical players within the ceRNA network may be exploited as novel therapeutic targets to enhance PD-L1-based cancer immunotherapy. The plasma level of exosomal Lnc-CCNH-8 also represents a novel predictive marker for immunotherapy response in HCC.

In lung cancer, a ceRNA network has been constructed by using immune-related genes of LUAD samples from the TCGA database^[98]. A 14-lncRNA immune-related signature was developed. In particular, the lncRNA C5orf64 was found to be positively correlated with the expression of immune checkpoint molecules (including PD-1, PD-L1, and CTLA-4) and immune cells (such as M2 macrophages, eosinophils, and neutrophils) but negatively associated with the immunosuppressive Tregs and plasma cells. Thus, the lncRNA C5orf64 may be used as an indicator to predict the status of tumor microenvironment (TME) modulation^[98]. The expression level of C5orf64 was found to be remarkably lower in LUAD tumor specimens than in non-tumorous tissues. Detailed ceRNA network analysis identified two potential pathways, C5orf64/miRNA-150/EGFR and C5orf64/miRNA-155-ITK, which presumably play a critical role in shaping an immunosuppressive TME^[98]. Further investigation is still needed to confirm the mechanistic explanation. The lncRNA FGD5-AS1 was reported to regulate PD-L1 expression by working as a miR-142 sponge, thereby promoting cancer cell proliferation, cisplatin resistance, migration, and tumor invasion^[99]. On the other hand, the antisense lncRNA NKX2-1-AS1 was shown to downregulate PD-L1 by modulating NKX2-1 protein expression and reducing the cellular expression of cell adhesion molecules (including E-cadherin), thereby inhibiting cell migration in lung adenocarcinoma^[100]. In NSCLC, the lncRNA MALAT1 was reported to upregulate PD-L1 by sequestering miR-200a-3p and promoting cancer propagation^[101]. Therefore,

FGD5-AS1/MALAT1 silencing or NKX2-1-AS1 ectopic expression may represent useful approaches to enhance PD-1/PD-L1 immunotherapy in lung cancer patients.

Furthermore, the dysregulation of circRNAs has also been reported to promote cancer development, migration, invasion, and immune evasion of NSCLC cells^[102]. For example, the high expression of circFGFR1 (derived from FGFR1) in NSCLC tissues is associated with poor prognosis and clinical outcomes^[103]. Mechanistically, circFGFR1 was shown to sequester miR-381-3p and increase the expression of its target gene CXCR4^[103]. CXCR4 genetic silencing could sensitize NSCLC cells to PD-1 blockade immunotherapy^[103]. To this end, CXCR4 is known to bind to CXCL12, subsequently increasing intracellular calcium content, cancer cell adhesion, proliferation, and gene transcription^[104,105]. Hong *et al.* recently reported that circCPA4 and PD-L1 were overexpressed, but the miRNA let-7 was expressed at a lower level in NSCLC cell lines (than in normal bronchial epithelial cells) and tumor specimens (than in adjacent normal lung tissues)^[106]. Importantly, NSCLC patients whose tumors expressed lower circCPA4 and PD-L1 levels but higher let-7 levels demonstrated a better prognosis. Detailed mechanistic investigation revealed that circCPA4 sponges let-7, thus upregulating PD-L1 to promote proliferation and EMT in NSCLC cells^[106]. Moreover, circCPA4 was also shown to increase exosomal PD-L1 levels. In the co-culture system of NSCLC (H1299 or A549) and CD8⁺ T cells (isolated from human PBMCs), the depletion of circCPA4 was shown to reactivate CD8⁺ T cells. Interestingly, PD-L1 depletion was also shown to increase the levels of IFN- γ and IL-4, but reduce IL-10 expression in the CD8⁺ T cells and the supernatants, suggesting that the NSCLC cells inactivated CD8⁺ T cells in the co-culture via secreting PD-L1^[106]. Collectively, circCPA4 increased PD-L1 expression in NSCLC cells by sponging let-7 to promote cancer stemness and cisplatin resistance, and also inactivated CD8⁺ T cells in the TME to facilitate immune evasion^[106].

Chen *et al.* recently reported the critical role played by the circRNA circNCOA3 in CRC immune escape and resistance to PD-1 blockade therapy^[107]. In CRC patient tumors, circNCOA3 was found to be remarkably overexpressed in samples resistant to anti-PD-1 monoclonal antibodies. High circNCOA3 was significantly correlated with worse survival and poor treatment outcomes in CRC patients. In cell line studies, the knockdown of circNCOA3 was shown to suppress cancer cell proliferation and invasion. In animal studies, circNCOA3 knockdown was found to increase the proportion of anticancer CD8⁺ T cells but reduce the abundance of the immunosuppressive myeloid-derived suppressor cells (MDSCs). Importantly, circNCOA3 knockdown was reported to retard tumor growth and potentiate the antitumor efficacy of PD-1 blockade immunotherapy in tumor-bearing mouse models^[107]. Subsequent mechanistic investigation revealed that circNCOA3 acted as a ceRNA for miR-203-3p to modulate CXCL1 expression. To this end, CXCL2 derived from the M2 macrophages, upon binding to CXCR2, has been shown to activate the PI3K/AKT/NF- κ B signaling pathway to increase PD-L1 expression. circNCOA3 may be further developed into a useful biomarker to predict the response and prognosis of CRC patients upon PD-1 blockade therapy.

In pancreatic cancer, hypoxia is known to induce HIF1, ADAM10, and sMICA, thus leading to reduced NKG2D in natural killer (NK) cells and immune escape of tumor cells^[108]. The circRNA circ_0000977 was induced by hypoxia. Knockdown of circ_0000977 was shown to potentiate NK cell-mediated lysis of cancer cells under hypoxic conditions in a HIF1- and ADAM10-dependent manner. Both HIF1 α and ADAM10 are direct downstream targets of miR-153. circ_0000977 was shown to sequester miR-153 and relieve the repression of HIF1 α and ADAM10 mRNA in pancreatic cancer cell line Panc-1. Collectively, the circ_0000977/miR-153/HIF1 α /ADAM10 ceRNA network represents a novel mechanism contributing to the hypoxia-mediated immune escape of pancreatic cancer cells^[108].

While there is growing interest in understanding the role of ceRNA regulation in cancer immunotherapy, research in this domain is still in its infancy. ceRNA regulation may also affect the expression of other immune-related genes regulating the TME, tumor infiltration of immune cells, and biological functions of

the immune cells. By modulating the expression of genes involved in various immune response pathways, ceRNAs could indirectly regulate the efficacy of cancer immunotherapy. It is noteworthy that the specific ceRNA networks and their impact on the various immune checkpoints may vary across different cancer types and in different contexts. Further research is needed to fully understand the complexity of ceRNA-mediated regulation of the immune checkpoints and its implications on cancer immunotherapy.

CONCLUSION AND FUTURE PERSPECTIVES

Understanding the ceRNA networks and their role in anticancer drug resistance could yield novel insights for the development of effective therapeutic strategies for treating refractory tumors and useful prognostic biomarkers for predicting clinical outcomes. By targeting specific ceRNAs or manipulating the ceRNA network, it may be possible to overcome drug resistance and improve the effectiveness of cancer treatments. Interestingly, flavonoids (bioactive polyphenolic compounds abundant in fruits, vegetables, and many medicinal plants) have been reported to regulate cancer-related genes via the ceRNA network, thereby inhibiting cancer growth and reversing chemoresistance^[109]. In gastric cancer, chrysin has been shown to promote apoptosis via the H19/miR-let-7a/COPB2 regulatory pathway^[110]. Another extensively studied flavonoid, quercetin, was reported to inhibit cancer proliferation and tumor invasion by upregulating miR-146a in breast cancer^[111] and sensitize NSCLC cells to radiotherapy by modulating the miR-16-5p/WEE1 axis^[112]. These ceRNA-modulating flavonoids may be combined with anticancer drugs to potentiate the therapeutic effect and overcome cancer drug resistance. Moreover, the ceRNA components involved may be exploited as useful biomarkers to select the patient population who could respond better to the drug combination.

Recent advances in high-throughput sequencing techniques have enabled the discovery of numerous biomarkers, including protein-coding RNAs and ncRNAs, from tissue and blood samples of cancer patients. To this end, most existing cancer biomarkers are solely relying on gene expression patterns. However, the expression patterns are not able to reflect the underlying interactions/mechanisms^[113,114]. Therefore, gene expression signatures for patient populations harboring the same clinical condition reported by different research teams are usually highly heterogeneous^[114]. To this end, system-guided ceRNA network analysis may represent a more reliable method for biomarker identification and the generated ceRNA signature may also facilitate precision medicine. The systematic method of computational approaches for ceRNA network-driven biomarker discovery has been recently reviewed^[115,116]. Clinical drug response analyses are usually performed to validate ceRNA biomarkers associated with anticancer drug responses^[117].

Components of ceRNA networks, including miRNAs, lncRNAs, and circRNAs, have been considered emerging biomarkers for cancer diagnosis, prognosis prediction, and treatment monitoring^[118-120]. Using a ceRNA network-driven method, a ceRNA network comprising 12 lncRNAs, 2 miRNAs, and 15 mRNAs was identified as a prognostic biomarker to predict survival for patients with pancreatic adenocarcinoma^[121]. For breast cancer, Wang *et al.* recently reported a lncRNA H19- and BRCA1/2-associated ceRNA signature that could distinguish patients with favorable versus dismal survival outcomes^[122]. For gastric cancer, Sui *et al.* identified a 2-lncRNA signature within a ceRNA network as a prognostic biomarker for predicting patient survival^[123]. The therapeutic significance is that genetic silencing of the two lncRNAs (LINC01644 and LINC01697) was shown to effectively inhibit gastric cancer cell proliferation. For lung cancer, a lung squamous cell carcinoma-specific ceRNA network has been recently constructed using TCGA RNA-sequencing datasets^[123]. A 2-lncRNA signature (consisting of FM06P and PRR26) was identified as a prognostic biomarker for overall patient survival^[123]. Moreover, ceRNA biomarkers have also been reported to predict and monitor anticancer drug response in the clinic. By integrating the expression profiles of lncRNA, miRNA, and mRNA from a pan-cancer ceRNA network with the patient survival data after anticancer drug treatment, Qi *et al.* identified a signature of drug response-related ceRNA (DRCE) that was

Table 5. Representative databases/online resources for ceRNAs relevant to anticancer drug resistance

Database	Online accession	Characteristic features	Ref.
Cupid	https://cupidtool.sourceforge.net/ (accessed on 22 May 2024)	● Online tool for simultaneous prediction of miRNA target interactions and their mediated ceRNA interactions	[6]
DIANA-LncBase v3.0	www.microrna.gr/LncBase (accessed on 22 May 2024)	● Collecting the correlations of miRNA-lncRNA pairs, and profiles of lncRNA expression in different cell types and organ tissues	[165]
ExoCeRNA atlas	https://ngdc.cncb.ac.cn/databasecommons/database/id/7334 (accessed on 22 May 2024)	● Composing cancer ceRNAs in human blood exosomes	[166]
lncCeDB	http://gyanxet-beta.com/lncedb (accessed on 22 May 2024)	● Listing human lncRNAs that may act as ceRNAs	[167]
Linc2GO	https://pubmed.ncbi.nlm.nih.gov/23793747/ (accessed on 22 May 2024)	● A human lincRNA function annotation resource based on ceRNA hypothesis ● Composing miRNA-lincRNA and miRNA-mRNA interaction data	[168]
LncACTdb 3.0	http://www.bio-bigdata.hrbmu.edu.cn/LncACTdb (accessed on 22 May 2024)	● Listing comprehensive information of ceRNAs in different species and under different disease states	[125]
LnCeVar	https://ngdc.cncb.ac.cn/databasecommons/database/id/6187 (accessed on 22 May 2024)	● Compiling genomic variations that may disrupt ceRNA network ● Curated from high-throughput sequencing datasets or published literature	[127]
LnCeCell	http://www.bio-bigdata.hrbmu.edu.cn/LnCeCell/ (accessed on 22 May 2024)	● Collecting cell-specific lncRNA-associated ceRNA networks, applicable for personalized characterization of diseases	[126]
miRSponge	https://bio.tools/mirsponge (accessed on 22 May 2024)	● Composing experimentally supported miRNA sponges and ceRNA networks	[169]
SomamiR 2.0	http://compbio.uthsc.edu/SomamiR (accessed on 22 May 2024)	● Compiling cancer somatic mutations in miRNA that may disrupt the interactions between miRNAs and ceRNA (circRNA, lncRNA, and mRNA)	[170]
StarBase v2.0	http://starbase.sysu.edu.cn/ (accessed on 22 May 2024)	● Collecting experimentally supported miRNA-lncRNA and miRNA-mRNA interactions ● Curated from CLIP-Seq data available in published literature	[171]

ceRNA: Competing endogenous RNA; miRNA: microRNA; lncRNA: long non-coding RNA; lincRNA: long intergenic non-coding RNA.

significantly correlated with individual drug response to cisplatin^[116]. Moreover, they also identified two DRCEs (NEAT1/hsa-miR-130b/TP53INP1 and NEAT1/hsa-miR-18a/NBR1) capable of modulating the anticancer efficacy of tamoxifen in breast cancer patients harboring TP53 mutation^[116]. Most recently, for lung cancer, Liao *et al.* reported a novel prognostic ceRNA network biomarker consisting of RGN (inhibitory protein of calcium signaling) and its related miRNA (hsa-miR-203a-3p) and two lncRNAs (ZNF876P and PSMG3-AS1)^[124]. The high RGN expression group was found to be associated with lower cancer immunotherapy efficacy and prognosis, which was consistent with an immunosuppressive tumor microenvironment^[125].

With the increasing number of experimentally validated ceRNA networks reported in recent years, a few databases have been developed to compile experimentally supported ceRNA interactions with comprehensive annotations [Table 5]. A few recently developed ceRNA databases also include patient demographics and clinical drug response data, thus making them more clinically relevant and potentially allowing for personalized prediction of ceRNA modulation outcomes. LncACTdb 3.0 is a comprehensive database of experimentally validated ceRNA interactions across 25 species and 537 diseases^[126]. It also compiled the lncRNA/mRNA/miRNA expression profiles with clinical and pathological information extracted from 62 datasets in TCGA and GEO. Computational tools are available for exploring the effects of ceRNA on individuals with specific pathological backgrounds.

LnCeCell is another database of predicted lncRNA-associated ceRNA networks constructed at the single-cell resolution^[127]. It is handpicked from cellular-specific ceRNA regulations and functional status of more than 94,000 cells in 25 tumor types. The database compiles more than 9,000 experimentally validated lncRNA

biomarkers, associated with drug resistance, prognosis, tumor metastasis, and recurrence. The unique feature of LnCeCell is that it provides a global map of ceRNA sub-cellular localization at a single cancer cell level, which was manually curated from the original data sources.

LnCeVar is another recently developed database of genomic variations that disturb ceRNA network regulation^[128]. It curated genomic variations-ceRNA events from patient samples and cell lines. Of relevance to anticancer drug response, the database contains more than 2,000 experimentally validated circulating, drug-resistant and prognosis-related lncRNA biomarkers. A few user-friendly searching and browsing interfaces are available for retrieval and analysis of data^[128]. In particular, LnCeVar-Survival can be used to conduct COX regression analyses and produce patient survival curves for specific genomic variation-ceRNA events. Thus, LnCeVar represents a useful tool for investigating the influence of personalized genomic variations that disturb ceRNA network in various diseases, including drug-refractory cancers.

It is noteworthy that the specific impact of disrupting ceRNA networks on cancer drug response may vary depending on the specific ceRNAs involved, the miRNAs and target genes regulated by the ceRNAs, and the cellular context. Subcellular localization and abundance of the ceRNA(s), and their interaction with other cellular pathways will also affect the biological outcomes of the ceRNA network. As the non-coding regions encoding regulatory RNAs make up close to 99% of the human genome, genomic alterations in cancer could have substantial effects on ceRNA networks that are largely regulated by ncRNAs. To this end, the intersection between cancer genomic alterations and ceRNA regulation has been unappreciated.

Moreover, an interplay between ceRNA network and epigenetic regulation of the miRNA components has been proposed^[129]. This is exemplified by the ceRNA network operating in the estrogen receptor signaling pathway. In ovarian cancer, miR-193a is known to target E2F6 (a downstream target of estrogen receptor), c-KIT (a widely-studied marker for cancer stemness), and PBX1 (a transcriptional activator for the immunosuppressive cytokine IL-10). Interestingly, epigenetic silencing of miR-193a by the E2F6 protein was shown to be required to upregulate c-KIT and PBX1 mRNA, in order to promote cancer stemness and immune evasion^[130]. Further research is warranted to fully unravel the complexity of ceRNA-mediated regulation and its implications on drug resistance.

DECLARATIONS

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Authors' contributions

Conceptualization, literature review, writing - original draft, review, and editing: To KKW, Cho WC
Writing - original draft, review, and editing: Zhang H
All authors read and approved the final manuscript.

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Conflicts of interest

To KKW and Cho WC are Editorial Board members of the journal *Cancer Drug Resistance*, while the other authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer-associated fibroblast-derived extracellular vesicles: regulators and therapeutic targets in the tumor microenvironment

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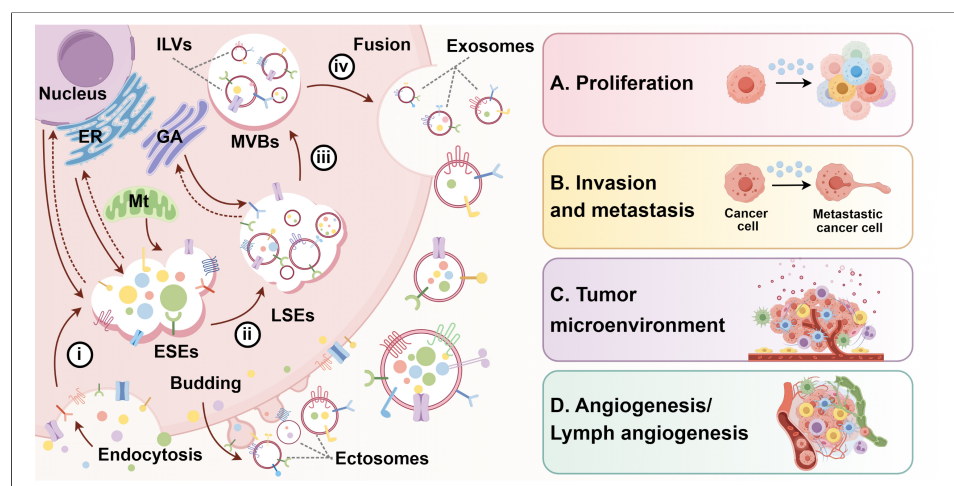
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Abstract

Cancer-associated fibroblasts (CAFs) constitute a critical component of the tumor microenvironment (TME). CAFs can be reprogrammed by cancer cells, leading to the production of extracellular vesicles (EVs). These EVs serve as carriers for bioactive substances, including proteins, nucleic acids, and metabolic products, thereby facilitating tumor progression. CAF-derived EVs exert substantial influence on tumor cell proliferation, invasion, and metastasis, the immunological environment, and the processes of lymphangiogenesis and angiogenesis. Despite their potential as non-invasive biomarkers and therapeutic delivery vehicles, the clinical application of CAF-derived EVs is currently limited by challenges in purification and precise targeting. This review delineates the diverse roles of CAF-derived EVs in tumor growth, metastasis, and immune evasion within the TME.



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INTRODUCTION

The tumor microenvironment (TME) refers to the internal milieu within which tumor cells proliferate and subsist, which consists of various cellular components and non-cellular components. Cellular components consist of immune cells and stromal cells. Among the immune cells are T and B lymphocytes, tumor-associated macrophages (TAMs), dendritic cells, natural killer cells, neutrophils, and myeloid-derived suppressor cells (MDSCs), and so forth. Stromal cells comprise tumor-associated fibroblasts [cancer-associated fibroblasts (CAFs)], pericytes, endotheliocytes, mesenchymal stromal cells, and the like; non-cellular components encompass extracellular matrix (ECM) and other secreted molecules such as growth factors, cytokines, chemokines, and extracellular vesicles (EVs), as well as the blood and lymphatic vascular network^[1]. The composition of the TME is quite complex and is a dynamic and variable process. It was once considered bystanders of tumorigenesis but is now known to play critical roles in the pathogenesis of cancer. However, mechanistic studies, including those involving preclinical tumor models, have shown that cancer development and progression occur alongside alterations in the surrounding stroma. Cancer cells and all other cells in the TME can functionally reprogram their microenvironment by secreting various cytokines, chemokines, and other factors, enabling them to play a determinative role in tumor survival and progression. The cellular composition and functional state of the TME can differ extensively depending on the organ in which the tumor arises, the intrinsic features of cancer cells, the tumor stage, and patient characteristics^[1-3]. In recent years, researchers have been exploring a new cancer treatment modality: targeting the tumor stroma.

CAFs are fibroblasts found within the TME near cancer cells and consist of multiple subtypes with distinct functions, demonstrating significant plasticity. Research suggests that CAFs are derived from different origins, such as pre-existing quiescent stellate cells and normal fibroblasts (NFs), bone marrow-derived fibroblasts and mesenchymal stem cells (MSCs), endothelial cells, epithelial cells, along with pericytes, smooth muscle cells, and adipocytes^[4]. Based on the expression of certain markers, classifications of CAFs broadly converge on three main subtypes: myofibroblastic CAFs (myCAFs), inflammatory CAFs (iCAFs), and antigen-presenting CAFs (apCAFs). These subtypes undergo alterations during tumor progression and are regulated at the spatial level. For instance, in pancreatic cancer, three distinct CAF subtypes coexist and possess different functional characteristics and transcriptomic plasticity. The functions of myCAFs and iCAFs rely on the secretion of ECM and immunomodulatory factors, respectively, while apCAFs interact directly with T cells to promote T cell exhaustion^[2,5]. CAFs are activated by inflammatory mediators within the TME, encompassing soluble factors generated by the tumor, such as transforming growth factor beta (TGF- β), interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- α)^[6,7]. Furthermore, cancer cells can transform dormant NFs into CAFs through direct cell-to-cell communication. For example, cancerous pre-mammary ductal cells can also activate CAFs in situ through direct epithelial-stromal interactions mediated by Jagged1/Notch2 ligand-receptor binding^[8]. Meanwhile, CAFs synthesize and remodel the ECM, modify the mechanical properties of the ECM, and alter the behaviors of cancer cells and immune cells. Additionally, CAFs have an impact on angiogenesis, possess robust immunomodulatory capabilities, and contribute to the immune evasion of cancer cells. CAFs interact extensively with cancer cells and can influence other components of the TME, such as the ECM and the immune infiltrates. Histopathological analysis has shown that the abundance of CAFs is related to prognosis among different human cancers^[6,7,9,10]. CAFs also modulate the efficacy of therapies and constitute a therapy target in their own right.

EVs are particles released from cells that are enclosed by a lipid bilayer and do not contain functional nuclei. EVs can be derived from a variety of eukaryotic cells, such as tumor cells, immune cells, and stem cells, as well as prokaryotic cells, including Gram-positive and Gram-negative bacteria. Moreover, EVs can be isolated from multiple body fluids and solid tissues, including blood, urine, cerebrospinal fluid, saliva,

synovial fluid, and brain or tumor tissues^[11,12]. EVs have a broad range of biological functions and participate in multiple physiological and pathological processes. Their ability to mediate intercellular communication by transferring a wide spectrum of molecules between cells gives them an important role in complex biological processes such as tumorigenesis, inflammation, immune response modulation, tissue repair, and apoptosis^[13-15]. In recent years, there has been growing evidence that CAFs are an important source of EVs in the TME, and CAF-derived EVs have been recognized as crucial mediators in regulating the extracellular communication between CAFs and cancer cells, and can affect tumor progression in multiple ways^[16,17].

OVERVIEW OF EVs

According to the Minimal information for studies of extracellular vesicles 2023 (MISEV2023) guidelines, EVs can be divided into different subtypes based on their size, density, molecular composition, or cell origin, among other characteristics. For example, EVs can be classified into small EVs and large EVs based on the diameter of the separated particles. Small EVs are often described as < 200 nm in diameter. Additionally, EVs can be classified into exosomes and ectosomes based on their biogenesis pathways. Exosomes are EVs from internal compartments of the cell that are released via the multivesicular bodies (MVBs), while ectosomes are EVs from the cell surface. Additionally, ectosomes are enriched for CD9 and CD81, while exosomes are enriched in CD63, CD9, CD81, Alix, and syntenin^[11,18,19]. Owing to the difficulty in isolating pure exosomes from cells in most instances and the ambiguity regarding the source of the isolated EVs, we term the exosomes mentioned in research articles “small Evs” or “Evs” rather than “exosomes”.

Synthesis and release of EVs

Exosomes originate from the endosomal pathway by the formation of the early sorting endosomes (ESEs), late sorting endosomes (LSEs), and ultimately MVBs, which contain intraluminal vesicles (ILVs). Finally, MVBs fuse with the plasma membrane, and exosomes are released. Through endocytosis and plasma membrane invagination, fluid and extracellular constituents such as proteins, lipids, metabolites, small molecules, and ions can enter cells, along with cell surface proteins. However, ectosomes are vesicles that pinch off the surface of the plasma membrane via outward budding, and include microvesicles, microparticles, and large EVs in the size range of 50 to 1 μm in diameter. During EV synthesis, donor cells' cytoplasm is loaded into EVs and secreted into the extracellular environment^[20-23] [Figure 1]. After successfully released into the extracellular environment, EVs can be taken up by interacting with receptors and ligands, thereby mediating material transport and information transmission between the TME and tumor cells^[24-26].

Contents and functions of EVs

EVs are highly heterogeneous, which is reflected in differences in their contents. EVs can carry various cargoes, including proteins, nucleic acids, and lipids, and this content can vary widely between cells and conditions. In addition to carrying bioactive contents, EVs are also involved in tumor cell proliferation, metastasis, drug resistance, and immune response^[27-29] [Figure 1].

Proteins are important components of EVs contents. There are multiple protein families in EVs, such as receptors, transcription factors, enzymes, and ECM proteins^[30]. EVs from different sources have different characteristics^[31]. Proteins in EVs from CAFs can participate in tumorigenesis by activating signaling pathways in recipient cells. For example, Sonic Hedgehog (SHH) in CAF-derived EVs can promote the proliferation and migration of esophageal squamous cell carcinoma (ESCC) through Hedgehog signaling^[32]. Additionally, Valbona Luga *et al.* found that tumor cells endocytose CD81-containing EVs from CAFs, which could activate the Wnt/β-catenin pathway to promote metastasis^[33].

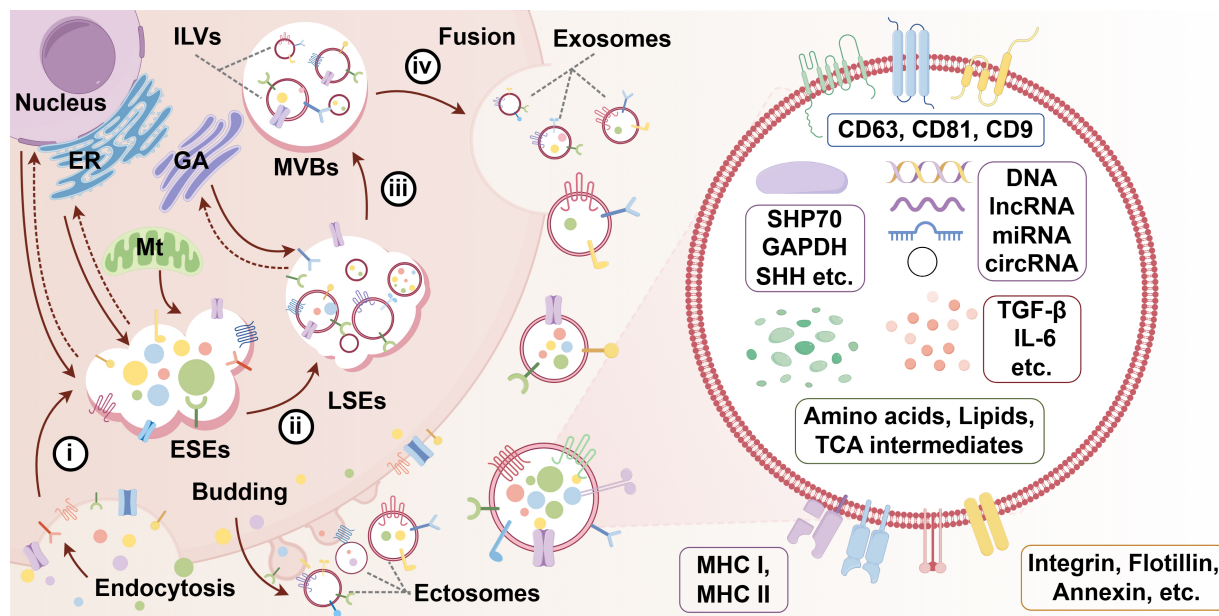


Figure 1. Biogenesis of extracellular vesicles and the structure and contents of exosomes. The formation of exosomes included four steps: (i) ESEs formation: Through the mechanisms of endocytosis and invagination of the plasma membrane, ESEs are formed, a process energized by Mt; (ii) LSE formation: After cytoplasmic sorting, ESEs mature and eventually form LSEs. During the formation of ESEs and LSEs, they are capable of exchanging materials with the cell nucleus, ER, and GA; (iii) MVB formation: LSEs' membrane buds inwardly to form multiple ILVs (future exosomes), which eventually transform into MVBs; (iv) Release: MVBs fuse with the plasma membrane, and ILVs released to extracellular space are called exosomes. Ectosomes are vesicles that pinch off the surface of the plasma membrane via outward budding. Exosomes have a diameter of 30-150 nm and a bilayer membrane structure, while ectosomes range from 50 to 1 mm in diameter. EVs are highly heterogeneous bilayer membrane structures carrying diverse cargos, such as proteins, nucleic acids, and lipids, and their content can vary significantly across different cells and conditions. ESEs: Early sorting endosomes; Mt: mitochondria; LSEs: late sorting endosomes; MVBs: multivesicular bodies; ILVs: intraluminal vesicles; ER: endoplasmic reticulum; GA: Golgi apparatus; SHH: Sonic Hedgehog; Evs: extracellular vesicles; TGF- β : transforming growth factor beta; IL-6: interleukin-6; TCA: tricarboxylic acid.

Accumulating evidence has recently shown that exosome-derived ncRNAs are vital for tumor progression^[34]. ncRNAs mainly include miRNAs, lncRNAs, and circRNAs. They mainly bind to the 3' untranslated region of target mRNA, inhibiting the translation and expression of target genes, thereby affecting the incidence and development of tumors^[35,36].

Metabolites derived from EVs affect the behavior of malignant tumors, and they can be divided into three major categories (1) amino acids: glutamine, threonine, serine, and valine; (2) lipids: palmitate and stearate; (3) tricarboxylic acid cycle intermediates: citrate, pyruvate, α -ketoglutarate, fumarate, and malate^[37]. Tumor metabolic reprogramming is one of the important features of cancer^[38], with the upregulation of glycolysis, glutaminolysis, lipid metabolism, mitochondrial biogenesis, pentose phosphate pathway, and other biosynthetic and bioenergetic pathways^[39], which leads to the rapid proliferation, survival, invasion, metastasis, and resistance to treatment of tumor cells^[40].

EVs from NFs and CAFs

In physiological conditions, fibroblasts are the quintessential supporting cell type and usually quiescent as shown by their low levels of cell proliferation and metabolic activity. They are present in all tissues, where they adapt to unique microenvironmental cues and cater to the needs of the specialized surrounding cells to help control tissue homeostasis and organ function. NFs in normal tissues produce ECM, which is vital to maintaining structural support and positional information for neighboring cells and offering a medium for cytokines, growth factors, and metabolites^[5,9,10]. During tissue injury or inflammatory response, fibroblasts can be activated, with enhanced cell proliferation and metabolic activity, including protein synthesis.

Activated fibroblasts are one of the key effector cell types in the wound healing process, which presumably promote wound healing via secreted factors including EVs^[41,42]. Oh *et al.* successfully isolated and purified L929-EV from fibroblasts. It can accelerate wound healing in the mouse model of skin trauma by promoting fibroblast proliferation and migration, along with endothelial cell migration and lumen formation^[43]. Xia *et al.*'s research found that EVs isolated from NFs at the wound edge of young mice were rich in miRNA-125b, which could accelerate myofibroblast differentiation and wound healing by inhibiting sirtuin 7^[44].

In certain conditions, the NFs can establish self-activating and feedback loops and transform into CAFs, which is crucial for the survival, proliferation, and invasion of cancer cells. For example, EVs secreted by CAFs differ in characteristics and biological functions from those secreted by NFs^[10]. Specifically, EVs from CAFs typically carry large amounts of inhibitory cytokines (e.g., TGF- β) and death receptor ligands (e.g., PD-L1)^[45]. Hu *et al.*'s study found that, compared with the EVs secreted by NFs, those secreted by CAFs increased expression of miR-92a-3p activates the Wnt/ β -catenin pathway and inhibits mitochondrial apoptosis by directly inhibiting FBXW7 and MOAP1, contributing to cell stemness, EMT, metastasis, and 5-FU/L-OHP resistance in colorectal cancer (CRC)^[46]. Additionally, Wang *et al.* found that in head and neck squamous cell carcinoma (HNSCC), CAF-derived EVs contain lower levels of miRNA-3188 compared to those derived from NFs^[47]. In the TME, CAF-derived EVs also regulate tumor growth, metastasis, and angiogenesis, and mediate therapy resistance of tumor cells.

REGULATORY MECHANISMS OF CAF-DERIVED EVS

CAF-derived EVs regulate tumor proliferation

The capacity for unlimited cell division is a cardinal feature distinguishing malignant tumor cell populations from their benign counterparts^[38]. Accumulating evidence suggests that ncRNAs, particularly miRNAs, regulate tumor cell proliferation through modulation of signaling pathways^[48] [Figure 2A]. For instance, miRNA-20a has been found to inhibit the PTEN/PI3K-AKT pathway in non-small cell lung cancer (NSCLC), which promotes the proliferation of tumor cells^[49]. Transfer of miRNA-500a-5p from CAFs to cancer cells in breast cancer (BC) stimulates proliferation and metastasis through binding to ubiquitin-specific peptidase 28 (USP28)^[50]. Furthermore, it has been reported that under nutrient deprivation or nutrient stress conditions, CAF-derived EVs might inhibit mitochondrial oxidative phosphorylation and enhance glutamine-dependent reductive carboxylation and glycolysis, which promotes tumor growth^[40].

In addition to promoting the proliferation, certain substances in EVs can also inhibit the proliferation. A study by Xu *et al.* discovered that miRNA-139 in gastric cancer (GC) can inhibit development and metastasis by reducing the levels of matrix metalloproteinase 11 (MMP11) in the TME^[51]. Wang *et al.* found that CAF-derived EVs in HNSCC contain lower levels of miRNA-3188 compared to those derived from NFs^[47]. Similarly, in hepatocellular carcinoma (HCC), miRNA-150-3p^[52] and miRNA-320a^[53] in EVs have been found to be significantly reduced. The reduction in or absence of these miRNAs has been shown to promote tumor proliferation, invasion, and metastasis. Therefore, the potential therapeutic value of CAF-derived EVs in inhibiting tumor growth warrants further investigation.

CAF-derived EVs regulate tumor invasion and metastasis

Metastasis is a significant cause of poor prognosis and mortality in cancer patients. It involves the dissemination of tumor cells to distant tissues and subsequent adaptation and survival in new microenvironments. Generally, the process of metastasis consists of four stages: local invasion and intravasation, survival in circulation, extravasation, and ultimately colonization at a new location^[54,55]. Among the most critical strategies for metastasis is the promotion of tumor dissemination through EV-ncRNAs [Figure 2B].

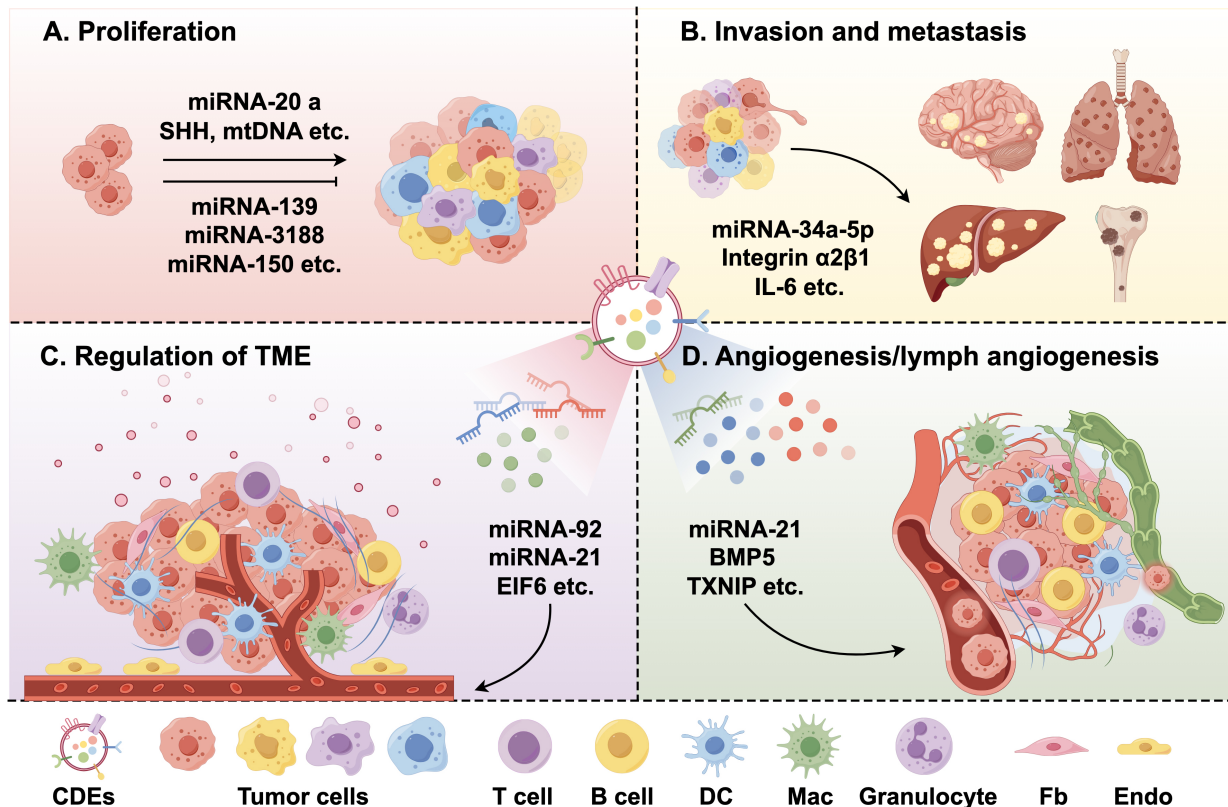


Figure 2. Contents and functions of CAF-derived EVs. (A) CAF-derived EVs regulate tumor proliferation; (B) CAF-derived EVs regulate tumor invasion and metastasis; (C) CAF-derived EVs regulate the tumor immune microenvironment; (D) CAF-derived EVs regulate tumor angiogenesis/lymphangiogenesis. CAF: Cancer-associated fibroblasts; EVs: extracellular vesicles; DC: dendritic cell; Mac: macrophage; Fb: fibroblast; Endo: endotheliocyte; TME: tumor microenvironment; IL-6: interleukin-6; CDEs: CAF-derived EVs.

In OSCC, the miR-34a-5p/AXL axis promotes β -catenin's nuclear translocation and induces the EMT, which in turn leads to SNAIL's transcriptional upregulation and the activation of MMP-2 and MMP-9^[56]. The upregulation of MMP expression accelerates cancer invasion and metastasis, as well as poor prognoses^[57]. Wu *et al.* found that the knockdown of the focal adhesion kinase (FAK) gene in CAFs promoted the migration of tumor cells. Analysis of miRNAs within CAF-derived EVs revealed multiple changes in EV-ncRNAs in FAK-deficient CAFs, such as the miRNA-16 and miRNA-148a. It is the upregulation of miRNA-16 and miRNA-148a that promotes the invasion and metastasis^[58]. However, the underlying mechanisms remain unclear.

Additionally, research indicates that in the early stages of cancer, CAF-derived EVs may facilitate the establishment of a pre-metastatic microenvironment (PMN), thereby enhancing the likelihood of metastatic tumor cells successfully surviving and colonizing in foreign microenvironments. In salivary adenoid cystic carcinoma (SACC), CAF-derived EVs with upregulation of plasma IL-6 and integrin β 1 possess robust stromal remodeling capabilities, inducing the formation of PMN in the lungs of mice and increasing pulmonary metastasis of SACC. Mechanistically, IL-6 activates the JAK2/STAT3 signaling pathway, which promotes EMT in SACC, thereby forming PMN to facilitate tumor metastasis^[59]. In contrast, integrin β 1 promotes the uptake of CAF-derived EVs by lung fibroblasts, further facilitating the formation of pulmonary PMN^[14]. These findings enhance our understanding of the mechanisms underlying tumor invasion and metastasis, offering novel insights for future research in the domain of CAF-derived EVs.

CAF-derived EVs regulate the TME

The inhibitory TME plays a pivotal role in determining the fate of tumors. During the initial stages of tumor development, the immune system can act as an antitumor defense. However, in later stages, tumor cells develop various mechanisms to evade immune surveillance. Despite recent advances in immunotherapy, the ability of tumors to evade immune surveillance has consistently been recognized as a barrier to the success of tumor immunotherapy [Figure 2C]^[60].

Researchers have shown that CAF-derived EVs can also contribute to the creation of an immunosuppressive microenvironment. Specifically, they can exhaust T cells in tumors and hinder their ability to perform essential immune functions. Feng *et al.* demonstrated that CAFs-EVs facilitate immune evasion in bladder cancer (BLCA) by upregulating the PD-L1/PD-1 pathway. Specifically, CAFs-EVs not only decrease apoptosis and enhance invasion in BLCA cells but also impair CD8⁺ T cell function by delivering PD-L1, thereby reducing CD8⁺ T cell proliferation and infiltration. Additionally, CAFs-EVs lower the secretion of key cytokines such as IFN- γ , IL-2, and TNF- α from CD8⁺ T cells^[61]. Furthermore, through STAT3 activation, miRNA-21 in CAF-derived EVs promotes the production of MDSCs. MDSCs are a type of immunosuppressive cells capable of inhibiting the proliferation of CD8⁺ T cells and contributing to the establishment of an immunosuppressive microenvironment^[62,63].

On the other hand, Wang *et al.*'s study revealed that EVs in oral squamous cell carcinoma (OSCC) not only reduce CD3⁺ and CD8⁺ immune cells in tumor tissue, but also shape an immunosuppressive microenvironment by influencing the expression of immune genes. CAF-derived EVs contain numerous substances related to immune regulation, including has-miRNA-139-5p, EIF6, and PECAM1 miRNA, which regulate the expression of target cell proteins, including PIGR, CD81, UACA, and PTTG1IP, in cancer-related pathways, playing a significant role in regulating immune responses and promoting OSCC growth^[64]. Currently, there is limited research on the impact of EVs on immune cells in the TME. Nevertheless, these studies provide a fresh perspective on the role of CAF-derived EVs in tumors and comprehensively reveal the immune microenvironment regulated by CAFs. Investigating the mechanisms of action of CAF-derived EVs in the tumor immune microenvironment represents a promising avenue for predicting prognosis and developing cancer treatment strategies.

CAF-derived EVs regulate tumor angiogenesis/lymphangiogenesis

Tumors proliferate rapidly and require new blood/lymphatic vessel networks to obtain nutrients for growth, but this can also increase the risk of metastasis^[65]. It has been demonstrated that CAF-derived EVs are crucial in regulating angiogenesis and lymphangiogenesis [Figure 2D].

In CRC, CAFs can downregulate the levels of BMP5 and TXNIP by upregulating the levels of miRNA-522-3p in small EVs^[66]. This promotes proliferation, migration, and invasion while inhibiting CRC cell apoptosis *in vitro*. Similarly, Wu *et al.* demonstrated that downregulation of miRNA-29b-1-5p in CAF-derived EVs via the VSIG1/ZO-1 axis inhibits angiogenesis, GC cell migration and invasion, as well as the progression^[67]. In the study conducted by Sun *et al.*, it is noteworthy that miRNA-21 is highly abundant in CAF-derived EVs. Moreover, miRNA-21 has been shown to significantly upregulate the expression of fibroblast activation protein and α -smooth muscle actin in NFs, thereby inducing their transformation into CAFs. The CAF-derived EVs play a crucial role in promoting angiogenesis in multiple myeloma (MM) by delivering miRNA-21 to MM endothelial cells^[68].

In addition to regulating angiogenesis, CAF-derived EVs are also closely linked to the development of lymphatic vessels. For example, ESCC primarily affects the stomach and intestines and has a high potential for early lymphatic metastasis. Compared to EVs from NFs, CAF-derived EVs show significantly lower levels

Table 1. The role and regulatory mechanism of CAF-derived EVs

Cancer type	Molecule	Expression	Mechanism	Function	Ref.
BC	miRNA-6, miRNA-148a	Upregulated	Through the FAK signaling pathway	Promotes invasion and metastasis	[58]
BC	miRNA-500 a-5p	Upregulated	Binds to USP28	Promotes proliferation and metastasis	[50]
BC	mtDNA	Upregulated	Restores oxidative phosphorylation of cancer cells, and activates cancer stem-like cells	Endocrine therapy resistance	[40]
CRC	miRNA-522-3p, BMP5, TXNIP	Upregulated	Inhibits the apoptosis, promotes angiogenesis	Promotes proliferation, migration, and invasion	[66]
ESCC	miRNA-100-5p	Downregulated	Inhibits lymphangiogenesis through IGF1R/PI3K/AKT axis	Inhibit lymphatic metastasis	[69]
ESCC	miR-21	Upregulated	Activates STAT3 signal transduction, induces MDSCs, inhibits CD8+ T cell proliferation	Establishes the immunosuppressive TME	[62,63]
ESCC	SHH	Upregulated	Activates the SHH signaling pathway	Promotes proliferation	[32]
GC	miRNA-139	Downregulated	Decreases MMP11 in the TME	Inhibits the metastasis	[51]
GC	miRNA-29b-1-5p	Upregulated	Promotes angiogenesis through the VSIG1/ZO-1 axis	Promotes proliferation, migration, and invasion	[67]
HCC	miRNA-150-3p, miRNA-320 a	Downregulated	Suppresses the MAPK pathway, inhibits EMT, decreases CDK2 and MMP2	Inhibits proliferation, migration, and invasion	[52,53]
HNSCC	miRNA-3188	Downregulated	Targets BCL2	Inhibits proliferation and promotes apoptosis	[47]
LC	Integrin $\alpha 2\beta 1$	Upregulated	Promotes lung fibroblast activation and the formation of lung PMN	Creates a pre-metastatic niche, promotes the metastasis	[14]
LC	miRNA-20 a	Upregulated	Inhibits the PTEN/PI3K/AKT pathway	Promotes the proliferation	[49]
MM	miRNA-21	Upregulated	Transforms NFs into CAFs, promotes tumor neovascularization	Promotes invasion	[68]
OSCC	has-miRNA-139-5p, EIF6, PECAM1 mRNA	Upregulated	Affects the expression of immune genes, reduces CD3 and CD8+ immune cells	Establishes the immunosuppressive TME and promotes the proliferation	[64]
OSCC	miRNA-34a-5p	Upregulated	Activates the AKT/GSK-3 β / β -catenin signaling pathway, induces EMT	Promotes progression and metastasis	[56,57]
SACC	Plasma integrin b1, IL-6	Upregulated	Activates the JAK2/STAT3 signaling pathway, induces EMT	Promotes invasion	[59]

BC: Breast cancer; BLCA: bladder cancer; CRC: colorectal cancer; ESCC: esophageal squamous cell carcinoma; GC: gastric cancer; HCC: hepatocellular carcinoma; HNSCC: head and neck squamous cell carcinoma; LC: lung cancer; MM: multiple myeloma; OSCC: oral squamous cell carcinoma; SACC: salivary adenoid cystic carcinoma; CDK2: cyclin-dependent kinase 2; BCL2: B-cell lymphoma 2; FAK: focal adhesion kinase; MDSCs: myeloid-derived suppressor cells; CAFs: cancer-associated fibroblasts; TME: tumor microenvironment; NFs: normal fibroblasts; PMN: pre-metastatic microenvironment; SHH: Sonic Hedgehog; USP28: ubiquitin-specific peptidase 28; MMP2: matrix metalloproteinase 2; MAPK: mitogen-activated protein kinase; EMT: epithelial-mesenchymal transition; PD-L1: programmed cell death ligand 1.

of miRNA-100-5p, which promotes the proliferation, migration, invasion, and lymphangiogenesis of tumor-associated lymphatic endothelial cells (TLECs). Mechanistically, miRNA-100-5p's inhibitory effect on lymphangiogenesis may be mediated through the IGF1R/PI3K/AKT signaling axis^[69]. Based on these findings, it has been observed that CAFs have the capability to secrete specific exosomes that regulate angiogenesis and lymphangiogenesis, thus opening up new avenues for research in this field.

Conclusively, CAF-derived EVs exert a pronounced regulatory influence on tumor initiation, progression, invasion, metastasis, immune evasion, and angiogenesis. The molecules, their mechanisms of action within these processes, and their biological functions are summarized in Table 1.

POTENTIAL CLINICAL APPLICATIONS OF CAF-DERIVED EVS

Innovative tumor biomarkers

EVs hold significant potential for liquid biopsy, as the cargo of EVs derived from tumor cells not only reflects the physiological and pathological characteristics of the tumor, but also remains stable in the body fluid circulation even within the harsh TME. Consequently, proteins and nucleic acids encapsulated in exosomes can serve as early diagnostic markers for cancer. For instance, miRNA-10b enclosed within exosomes is a well-established indicator of pancreatic adenocarcinoma (PAAD) progression and is widely used for early diagnosis. Furthermore, Glypican-1 (GPC1) is specifically enriched in exosomes, and circulating exosomes with high GPC1 expression can be highly specific and sensitive when detected in the serum of PAAD patients, distinguishing between early-stage and late-stage patients with PAAD as well as healthy individuals with pancreatic diseases^[70]. For example, Tian *et al.* analyzed the cancer-related proteome of plasma EVs from BC patients and found that the EV features were highly accurate in differentiating between metastatic and non-metastatic BC. It can precisely monitor the response to treatment and serve as an independent prognostic factor in metastatic breast cancer (MBC) patients^[71]. The development of biomarkers based on EVs is intrinsically linked to the advancement of EV detection technology. When contrasted with traditional EV analysis methods, such as polymerase chain reaction (PCR), western blot (WB), EV detection methods based on fluorescence, surface-enhanced Raman spectroscopy (SERS), surface plasmon resonance (SPR), electrochemistry, and aptamers enhance the sensitivity, specificity, and practicality of EVs in clinical settings^[72,73]. In conclusion, as technology progresses and research intensifies, EVs will exhibit an even more significant potential in cancer.

Drug delivery system in cancer therapy

In the field of cancer treatment, EVs are capable of enhancing the targeting capacity of cells or organs through genetic engineering and chemical modification and transferring various cargos to the target cells. The EV-mediated drug delivery technology is categorized into two types: exogenous and endogenous. Exogenous drug delivery technology refers to the isolation and purification of exosomes from cell culture supernatants or other biological fluids and the loading of the desired drugs onto the surface or within exosomes by physical or chemical means^[74]. For instance, Santos *et al.* loaded exogenous miRNA-195-5p onto EVs. These modified EVs were absorbed by tumor cells and could reduce cell proliferation, increase cell death, and enhance targeted therapy responses in melanoma patients^[74,75]. Exogenous loading is applicable when the ideal carrier is a small molecule drug or other molecules that cells cannot produce. The modification methods for EVs include passive incubation with cargo or active loading through electroporation, sonication, or freeze-thaw cycles. Meanwhile, endogenous drug delivery technology encompasses modifying donor cells via biotechnology to yield exosomes with specific products. This approach enables more precise control of the carrier content and has been employed to load therapeutic proteins, RNA, and CRISPR/Cas9 complexes into EVs^[74]. For example, Katakowski *et al.* utilized a miRNA-146b plasmid to transfect MSCs, resulting in exosomes carrying it, which were then administered into the glioblastoma sites in mice. Their findings indicated that exosome-based therapy utilizing miRNA transport could effectively impede tumor cell proliferation^[76]. Furthermore, numerous methods have been devised for the loading of siRNA into EVs, such as calcium chloride-mediated transfection, electroporation, sonication, and the employment of hydrophobically modified siRNA. For instance, in the study by Zhang *et al.*, L9-29 mouse fibroblasts were transfected with TGF- β 1 siRNA through a transfection reagent, and exosomes with TGF- β 1 siRNA were isolated from the cell culture supernatant. The findings showed that compared with the use of free TGF- β 1 siRNA, EVs carrying TGF- β 1 siRNA effectively reduced the level of TGF- β 1 in the targeted tumor cells, significantly lowering the viability and migration ability of mouse sarcoma cells^[77]. The engineered CAF-derived EVs investigated by Xue *et al.* exhibit robust intercellular communication, transport, penetration, and targeting properties, making them a highly efficient delivery system capable of effectively overcoming the complexity and heterogeneity of the TME^[78]. Similarly, there is

significant potential in blocking tumor progression by regulating the secretion or level of CAF-derived EVs. For instance, the study by Sun *et al.* demonstrated that the application of anti-miRNA-21 or siRNA technology to lower the miRNA-21 level in CAF-derived EVs could diminish the proliferative, invasive, and metastatic capabilities of tumor cells *in vitro*^[68]. Although the research targeting CAF-derived EVs for tumor treatment is still primarily at the animal experiment stage, it remains a potential target for cancer therapy.

Advantages and disadvantages of EVs in clinical application

EVs have shown advantages and potential in clinical applications. Firstly, EVs have a natural targeting ability, as different cell-derived EVs interact with receptor carriers through surface adhesion proteins and selectively target receptor cells through their membrane components. For example, integrin $\alpha 6 \beta 4$ and $\alpha 6 \beta 1$ can target EVs to the lungs, while integrin $\alpha 6 \beta 5$ can promote EV targeting to the liver^[79,80]. Secondly, EVs can be derived from the patient's own cells, such as MSCs, thereby reducing their risk of immune rejection. Moreover, EVs are incapable of self-replication, which might render them safer than stem cell transplantation in regenerative medicine^[81,82]. EVs possess distinctive advantages as biomarkers for therapeutic responses or drug delivery systems. EVs encompass various biological macromolecules and play a crucial role in information transmission among different cell types. They exhibit advantages such as stability, rich content, minimally invasive sampling, higher concentrations, and enhanced stability in liquid samples, as well as existence in various types of liquid samples. This renders EVs a promising new circulating biomarker with extensive application prospects^[72,83]. Additionally, EVs possess the following advantages as drug delivery vehicles. Firstly, compared to cell therapy, exosomes are safer and easier to store, and they can be separated from the patient's body fluids and modified before reintroduction, significantly reducing the likelihood of immune reactions occurring in clinical settings^[84,85]. Secondly, exosomes possess the ability to enter the cell cytosol and protect drugs (such as nucleic acids) from metabolic processes during transportation, thereby extending the drug's circulation time and improving its stability^[84]. Thirdly, exosomes are nanometer-sized vesicles that carry cell surface molecules, giving them strong penetration and inherent targeting capabilities across various biological barriers^[86].

However, EVs have disadvantages in clinical applications. First, the half-life of EVs in body circulation is short, which might affect their sustained efficacy. The impact of EVs on tissues depends on their clearance rate, and macrophages have a certain clearance effect on EVs^[15,83,87]. Regarding production efficiency, for regenerative therapy, EV production requires cultivating a large number of MSCs; however, the output of EVs secreted by cells is too low to meet therapeutic needs^[81,82]. In terms of separation, purification, and storage, EVs have certain limitations. The currently mature methods for EV separation and purification include ultracentrifugation, immunoprecipitation or affinity purification, ultrafiltration, and size exclusion; however, the purity of purified EVs is often low, affecting subsequent experiments. The optimal storage conditions for maintaining EVs' integrity (such as temperature or pH value) are rather demanding^[15,72,83]. Nonetheless, EVs continue to demonstrate significant potential for precision cancer treatment in the future.

CONCLUSION

CAF-derived EVs have a multifaceted and pivotal impact on the TME. This comprehensive review outlines the diverse functions of CAF-derived EVs in tumorigenesis, progression, metastasis, and immune evasion. It also investigates their potential applications in cancer therapy.

Firstly, CAF-derived EVs influence tumor cell proliferation, invasion, and metastasis by transporting various biologically active molecules such as proteins, nucleic acids, and metabolic products. Notably, ncRNAs such as miRNAs and lncRNAs modulate the expression of target genes, enhancing tumor cells' stem-like properties and EMT while improving chemoresistance and metastatic potential. Additionally, metabolic products in CAF-derived EVs contribute to reprogramming tumor metabolism by providing energy and

precursor molecules for tumor growth and invasion. Secondly, CAF-derived EVs play a critical role in regulating the TIME. They can impact T cell function, facilitate MDSCs generation, and regulate the expression of immune-related genes to establish an immunosuppressive microenvironment that aids tumors in evading immune surveillance. Furthermore, the role of CAF-derived EVs in tumor angiogenesis and lymphangiogenesis warrants significant attention. By delivering specific miRNAs, CAF-derived EVs can modulate the expression of factors associated with angiogenesis, thereby impacting the vascular supply and metastatic potential of tumors.

In terms of clinical application, circulating CAF-derived EVs have shown promise as novel biomarkers, owing to their stability in bodily fluids and ability to reflect tumor characteristics through specific proteins and nucleic acids. Additionally, CAF-derived EVs can function as delivery vehicles to effectively transport therapeutic molecules to tumor cells. However, improving the targeting and purity of CAF-derived EVs remains a significant challenge in realizing their clinical utility. Lastly, it is possible to inhibit tumor proliferation and metastasis by modulating the secretion of CAF-derived EVs or the levels of their constituents, thereby providing a new avenue for therapeutic intervention.

In summary, the role of CAF-derived EVs in tumor biology is multifaceted, with broad prospects for their application in tumor diagnosis and treatment. Future research needs to further elucidate the specific mechanisms of action of CAF-derived EVs, optimize their use as biomarkers and drug carriers, and explore new targeted therapies for more effective cancer treatments.

DECLARATIONS

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Authors' contributions

Provided direction and guidance throughout the preparation of this manuscript: Xie X, Chen W, Zou Y

Wrote and edited the manuscript: Xie J, Lin X, Deng X

Reviewed and made significant revisions to the manuscript: Xie J, Lin X, Deng X, Tang H

All authors confirm that they contributed to manuscript reviews, critical revision for important intellectual content, and have read and approved the final draft for submission.

All authors agree to be accountable for the content of the work.

Availability of data and materials

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Conflicts of interest

Tang H is an Associate Editor on the Editorial Board of the journal *Cancer Drug Resistance*. Zou Y is an Editor on the Junior Editorial Board of the journal *Cancer Drug Resistance*. Tang H and Zou Y were not involved in any steps of editorial processing, notably including reviewer selection, manuscript handling and decision making. The other authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cisplatin resistance in head and neck squamous cell carcinoma is linked to DNA damage response and cell cycle arrest transcriptomics rather than poor drug uptake

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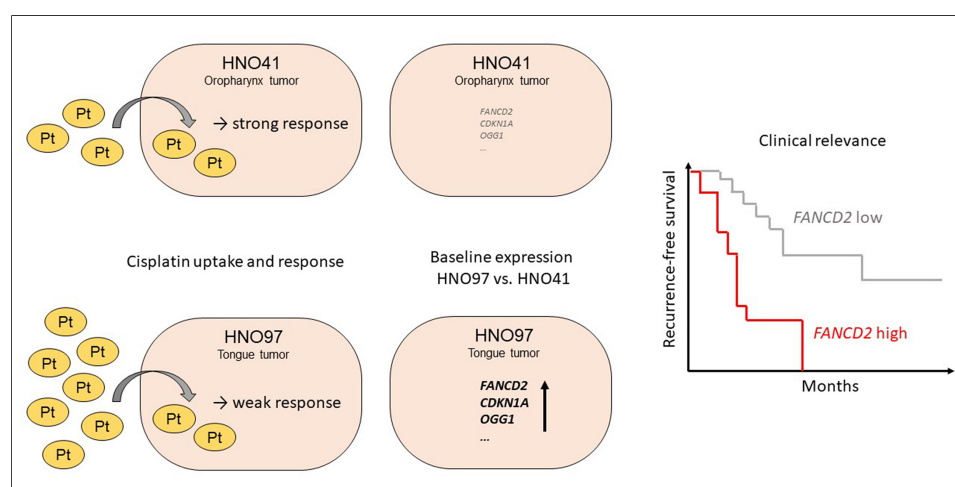
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Abstract

Aim: Cisplatin resistance in head and neck squamous cell carcinoma (HNSCC) is thought to involve both reduced drug uptake and altered molecular responses. However, the relative contribution of these mechanisms remains unclear.

Methods: Two HNSCC cell lines with differing sensitivity (HNO97 and HNO41) were analyzed using cytotoxicity assays, atomic absorption spectroscopy-based quantification of intracellular cisplatin, caspase 3/7 assays, Western blotting, polymerase chain reaction (PCR)-based transcriptomic analysis of DNA damage response and cell cycle arrest pathways, and RNA-seq data from The Cancer Genome Atlas (TCGA) to characterize the resistance phenotype.

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Results: HNO97 ($IC_{50} = 440 \mu M$) was 7.6-fold more resistant to cisplatin than HNO41 ($IC_{50} = 57.8 \mu M$; $P = 0.0286$). After quantifying intracellular uptake (pg Pt/ μg protein) and normalizing cytotoxicity to intracellular drug levels, HNO97 ($IC_{50} = 778.9$ pg Pt/ μg protein) remained 5-fold more resistant than HNO41 ($IC_{50} = 153.5$ pg Pt/ μg protein), indicating only a partial reduction in resistance (33% decrease, from 7.6-fold to 5-fold; $P = 0.0286$). At cisplatin concentrations yielding comparable intracellular exposure (HNO97: $440 \mu M$; HNO41: $196 \mu M$; both ≈ 725 pg Pt/ μg protein), caspase 3/7 activation and induction of *CDKN1A*, *GADD45A*, *GADD45G*, and *PPP1R15A* were weaker in HNO97 than in HNO41. Notably, baseline expression of these genes was significantly higher in HNO97. In the TCGA cohort, multivariate analysis showed that high *FANCD2* expression was associated with unfavorable recurrence-free survival in platinum-treated patients (hazard ratio = 4.0; $P = 0.011$), but not in those who did not receive platinum chemotherapy.

Conclusion: Cisplatin resistance in HNSCC appears to be driven primarily by molecular mechanisms involving DNA damage response and cell cycle arrest pathways, rather than poor drug uptake.

INTRODUCTION

Cisplatin remains a cornerstone in the treatment of head and neck squamous cell carcinoma (HNSCC), whether used as monotherapy, in combination with other agents, or alongside radiotherapy^[1-3]. However, treatment efficacy - and consequently long-term survival - remains unsatisfactory, largely due to intrinsic or acquired resistance to cisplatin in HNSCC^[4]. Extensive research has investigated the mechanisms underlying cisplatin resistance in HNSCC, which can generally be divided into two major categories^[5,6]. The first involves reduced cisplatin uptake and limited intracellular accumulation^[7]. Besides passive diffusion, cisplatin enters and exits cells via various transmembrane drug transporters. Altered expression or activity of these transporters can significantly influence cellular drug levels and thereby confer resistance^[7].

The second mechanism relates to altered molecular responses to cisplatin. Among the many factors implicated, the upregulation of genes and proteins involved in DNA damage response pathways is particularly important. For instance, enhanced repair of platinum-DNA adducts by the nucleotide excision repair machinery (e.g., *ERCC1*, *XPF*) or the Fanconi anemia/BRCA pathway (e.g., *FANCD2*, *BRCA1*) has been well documented and linked to cisplatin resistance in various tumor types^[6,8], including HNSCC^[9-14]. Moreover, genes involved in cell cycle regulation and tumor suppression (e.g., *CDKN1A*) have also been associated with resistance in HNSCC^[15-17]. Taken together, cisplatin resistance in HNSCC reflects a multifaceted interplay between reduced drug uptake and altered molecular responses. The relative contribution of these mechanisms, however, remains unclear and was therefore systematically evaluated and quantified in this study.

METHODS

Materials

Cell culture flasks and multi-well plates were obtained from Greiner (Frickenhausen, Germany). Dulbecco's Modified Eagle Medium (DMEM) and fetal calf serum (FCS) were purchased from PAN-Biotech (Aidenbach, Germany). Phosphate-buffered saline (PBS), penicillin-streptomycin (100 $\times$), and the GeneElute Mammalian Total RNA Miniprep Kit were purchased from Sigma-Aldrich (Taufkirchen, Germany). The Pierce™ BCA Protein Assay Kit and Pierce™ Detergent Compatible Bradford Reagent were obtained from Thermo Scientific (Rockford, USA). Laemmli buffer was obtained from Bio-Rad (Munich, Germany). Antibodies for Western blotting were purchased from Cell Signaling Technology (Danvers, USA). Luminata

Forte ECL reagent was obtained from Millipore (Darmstadt, Germany). The RT² First Strand Kit and RT² polymerase chain reaction (PCR) DNA Damage and Signaling Pathway Arrays were purchased from Qiagen. The Caspase-Glo 3/7 Assay was obtained from Promega (Mannheim, Germany). Cisplatin (1 mg/mL stock in physiological (0.9%) sodium chloride) was provided by the Heidelberg University Hospital pharmacy.

Cell lines

The HNSCC cell lines HNO97 (tongue, T3N2bM0, histologic grade 4) and HNO41 (tonsil/oropharynx, T2N2bM0, histologic grade 2) were established from intraoperatively obtained samples and have been extensively characterized^[18]. Cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate.

Viability assay

Cell viability was assessed using crystal violet staining, as previously described^[19]. Cells were seeded at a density of 3×10^4 cells per well in 96-well microtiter plates and allowed to attach for 24 h at 37 °C. The first column of wells contained no cells and was used to measure background absorbance. The second column contained untreated cells, representing 100% viability. All other columns were exposed to varying concentrations of cisplatin (1 to 1,000 µM). Each concentration was tested in octuplicate, and each assay was repeated four times for both cell lines. After a 2.5-h incubation, the medium in all wells was replaced with drug-free medium, and cells were further incubated for 24 h at 37 °C. The next day, cells were washed once with PBS and stained with 50 µL of crystal violet solution (0.5% w/v in 20% methanol). Wells were then washed three times with tap water to remove unbound dye and dried at 37 °C. Crystal violet was solubilized by adding 200 µL of pure methanol to each well, followed by shaking for 10 min at room temperature. Absorbance was measured at 555 nm using a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices). For quantification, the mean absorbance of cell-free wells was subtracted from each sample. Cell viability was calculated as the ratio of absorbance in drug-treated wells to that in untreated wells (100% viability). Sigmoid concentration-response curves and IC₅₀ values were generated using GraphPad Prism version 9 (GraphPad Software Inc., La Jolla, USA). Fold-change in cisplatin resistance was determined by normalizing replicate IC₅₀ values of HNO97 to the mean IC₅₀ of HNO41.

Cellular platinum quantification in cisplatin-treated cells

Cells were seeded at a density of 1×10^6 cells per well in 6-well plates (six replicates) and preincubated at 37 °C for 24 h. The cells were then treated with the same cisplatin concentrations used in the proliferation assay. After a 2.5 h exposure, the drug-containing medium was discarded, and the cells were washed once with PBS. Cells were harvested by scraping in 150 µL of 0.4% HNO₃ and lysed by sonification for 30 min. The lysates were centrifuged at $17,000 \times g$ for 3 min, and the supernatants were collected for platinum quantification using a PinAAcle 900Z graphite furnace atomic absorption spectrometer (PerkinElmer). Platinum detection was based on the background-corrected absorption peak at 265.94 nm following injection of a 20 µL sample into the furnace. The following atomic absorption spectroscopy program was used: 30 s at 110 °C, 30 s at 130 °C, 20 s at 1,300 °C, 5 s at 2,200 °C, and 3 s at 2,450 °C. A weighted linear calibration curve was constructed, using five calibration standards spanning the expected concentration range. Three quality control samples (low, middle, and high concentrations) were included. All calibration standards, quality controls, and experimental samples were measured in duplicate and were required to meet US Food and Drug Administration (FDA) criteria for accuracy and precision to be included in the final analysis^[20].

Platinum concentrations were normalized to the protein content of each sample, determined with a commercial bicinchoninic acid (BCA) assay kit. Briefly, a calibration curve was prepared using nine concentrations of bovine serum albumin (BSA, 0–2,000 µg/mL), dissolved in the same cell lysis buffer (0.4% HNO₃). Wells of a 96-well plate were loaded with 8 µL of either BSA standards or supernatants, along with

64 μ L of the working solution (a mixture of reagents A and B provided in the kit). After 30 min of light-protected incubation at 37 °C, absorbance was measured at 562 nm using a SpectraMax plate reader (Molecular Devices, München, Germany).

Caspase 3/7 assay

Caspase 3/7 activity in response to similar intracellular platinum concentrations was assessed in HNO97 and HNO41 cells using the Caspase-Glo 3/7 Assay (Promega, Mannheim, Germany). Cells were seeded at 3×10^4 cells per well in white-bottom 96-well plates and incubated for 24 h at 37 °C. Based on the known cisplatin uptake characteristics of HNO97 and HNO41, cells were treated with extracellular cisplatin concentrations previously validated to yield similar total intracellular platinum levels. After 2.5 h of exposure, the medium was replaced with 50 μ L of fresh medium. Caspase 3/7 activity was then measured at the following time points: 0 h (immediately after the 2.5-h treatment) and 4, 8, 12, 18, and 24 h post-treatment. Prior to reagent addition, both the plates and the detection reagent were equilibrated to room temperature. Then, 50 μ L of reagent was added to each well, the plates were shaken for 30 s on a rotary shaker, and subsequently incubated for 30 min at room temperature. Luminescence was measured using a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, Wokingham, UK), and caspase 3/7 activity was normalized to the mean of untreated control wells at the corresponding time point.

Western blotting

Cleavage of Caspase 3 and PARP was assessed 4 h after the 2.5-h cisplatin treatment. Cells were seeded at 2×10^6 cells per T25 flask (four independent experiments) and incubated overnight at 37 °C. They were then treated for 2.5 h with cisplatin concentrations validated to produce comparable total intracellular platinum levels. Following drug exposure, cells were transferred to drug-free medium for 4 h. The medium was then removed, cells were washed with PBS and harvested by trypsinization. The resulting pellets were washed again with PBS and subjected to western blot analysis: Cell pellets were lysed in hot SDS lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1% (m/v) Sodium Dodecyl Sulfate (SDS), 1 $\times$ protease inhibitor cocktail, and 1 $\times$ PhosSTOP phosphatase inhibitor. Protein concentration was measured using the Pierce Detergent Compatible Bradford Reagent (#23246, Thermo Fischer Scientific). Lysates were mixed with Laemmli buffer (#1610747, Bio-Rad, Munich, Germany), heated, and 20 μ g of protein per sample was separated on a 12% SDS-PAGE gel and transferred to PVDF membranes. Membranes were blocked in 5% (w/v) milk/PBS-T and incubated overnight at 4 °C with the following primary antibodies (Cell Signaling Technology): cleaved caspase 3 (#9661), PARP (#9542), and GAPDH (#2118). After incubation with the secondary antibody (Cell Signaling Technology, #70745), proteins were visualized using Luminata Forte ECL (Millipore, Darmstadt, Germany).

PCR array-based transcriptomic analysis

Based on the caspase assay results, the optimal time point for gene expression analysis was determined to be 4 h after the 2.5-h cisplatin treatment phase. Cells were seeded at 2×10^6 per T25 flask (three independent experiments) and incubated overnight at 37 °C. They were then treated again for 2.5 h with cisplatin concentrations previously validated to yield comparable total intracellular platinum levels. Following drug exposure, cells were transferred to drug-free medium for 4 h. The medium was then removed; cells were washed with PBS and harvested via trypsinization. RNA was isolated from the resulting cell pellets using the GenElute™ Mammalian Total RNA Kit (Sigma, Taufkirchen, Germany) according to the manufacturer's protocol. cDNA synthesis was performed using the RT² First Strand Kit (Qiagen, Hilden, Germany). The resulting cDNA was analyzed on a LightCycler 480 (Roche, Mannheim, Germany) using the RT² PCR DNA Damage and Signaling Pathways Array. The RT² Profiler PCR Arrays are designed to analyze panels of genes related to specific disease states or biological pathways. Each array contains primer pairs for 84 target genes and five housekeeping genes (β -actin, β 2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase,

hypoxanthine phosphoribosyltransferase 1, and large ribosomal protein). In addition, one well contains a genomic DNA control, three contain reverse-transcription controls, and three contain positive PCR controls. Data analysis was performed using Qiagen's online PCR data analysis tool, which applies the widely used $\Delta\Delta C_t$ method^[21] with a combination of housekeeping genes. *P* values were calculated using Student's *t*-test on replicate $2^{-\Delta\Delta C_t}$ values for each gene in the test groups compared with the control group.

TCGA data evaluation and survival analysis

Data were obtained from The Cancer Genome Atlas (TCGA) Head and Neck Squamous Cell Carcinoma (HNSC) dataset, accessed through the Broad Institute's Firehose repository. Clinical data, including survival information (recurrence-free survival time and status) and drug treatment details, were extracted and curated. Patients were filtered to include only those with complete survival data and corresponding RNA-Seq profiles. RNA-Seq count data were normalized using the voom function from the limma R package. For each gene/signature of interest, normalized expression values were dichotomized at the cohort median to define "high" and "low" expression groups. Recurrence-free survival between these groups was compared using Kaplan–Meier estimation and the log-rank test. Resulting *P*-values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate procedure. Genes exhibiting at least a suggestive association in univariate analyses ($P < 0.10$) were further assessed using multivariable Cox proportional hazards models to adjust for potential clinical confounders. Survival analyses were conducted using the survival R package. Statistical significance was determined using the log-rank test for univariate analysis and the Cox proportional hazards regression model for multivariable analysis.

Statistical analysis

Cytotoxic IC_{50} values were compared using the non-parametric Mann-Whitney test. Intracellular platinum concentrations following drug treatments were also compared using the Mann-Whitney test. Differences in caspase 3/7 activity at specific time points were evaluated by Student's *t*-test. All statistical analyses and figure generation were performed using GraphPad Prism version 9.0.0. (Boston, USA).

RESULTS

Cytotoxic pharmacodynamics of cisplatin

When calculated based on nominal cisplatin concentrations in the culture medium, the IC_{50} for HNO41 cells was $57.8 \pm 1.44 \mu M$. In contrast, HNO97 cells were 7.6-fold (± 0.94) more resistant ($IC_{50} 440 \pm 54.3 \mu M$; $P = 0.0286$; [Figure 1A](#)). However, when cytotoxic effects were normalized to total intracellular platinum concentrations, HNO97 cells ($IC_{50} 778.9 \pm 115.9 \text{ pg Pt}/\mu\text{g protein}$) were only 5-fold (± 0.75) more resistant than HNO41 cells ($IC_{50} 153.5 \pm 4.35 \text{ pg Pt}/\mu\text{g protein}$; $P = 0.0286$), corresponding to a $33\% \pm 0.1\%$ ($P = 0.0286$) decrease in the difference in cisplatin resistance [[Figure 1B](#)].

Caspase 3/7 kinetics in cells exposed to comparable total intracellular platinum concentrations

By comparing the nominal cisplatin concentrations in the culture medium with the resulting total intracellular platinum concentrations in the two cell lines, uptake characteristics could be described using linear regression. Overall, HNO97 showed reduced drug uptake compared with HNO41, as indicated by the significantly different slopes of the regression lines (HNO97 slope: 2.27 ± 0.04 ; HNO41 slope: 5.1 ± 0.11) [[Figure 2](#)]. Based on these linear uptake characteristics, cisplatin concentrations of $196 \mu M$ (HNO41) and $439.5 \mu M$ (HNO97) were selected to achieve similar total intracellular platinum levels ($725 \pm 54.3 \text{ pg Pt}/\mu\text{g protein}$; $P > 0.9999$; [Figure 3A](#)). At equivalent intracellular drug exposure, caspase 3/7 activity was monitored over time. In HNO41 cells, caspase 3/7 activity was already markedly elevated 4 h after treatment (5.5-fold compared with control), whereas no increase was observed in HNO97 at the same time point ($P = 0.0008$). Caspase 3/7 activity peaked at 8 h in HNO41 (8-fold compared with control) but was delayed until 12 h in HNO97 (7-fold compared with control) [[Figure 3B](#)]. To confirm the reduced pro-apoptotic and DNA

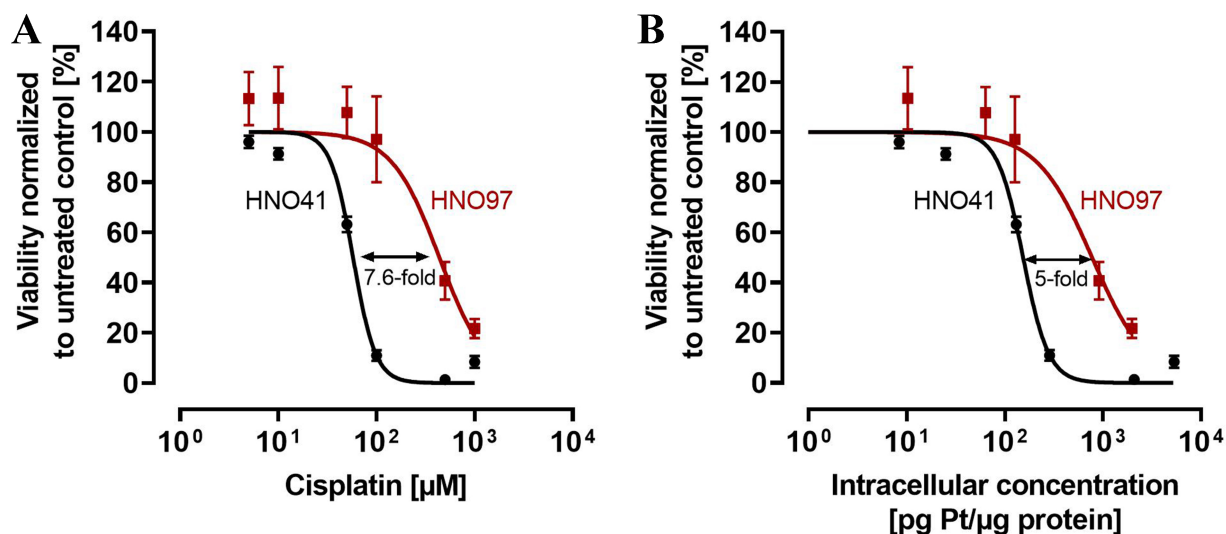


Figure 1. Cytotoxic pharmacodynamics of cisplatin in HNO41 (black) and HNO97 (dark red). (A) Cytotoxic effects of cisplatin as a function of nominal concentrations in the culture medium; (B) Cytotoxic effects of cisplatin normalized to total intracellular platinum concentrations. Data are presented as mean $\pm$ SD from four independent biological replicates, each with eight technical replicates. SD: Standard deviation.

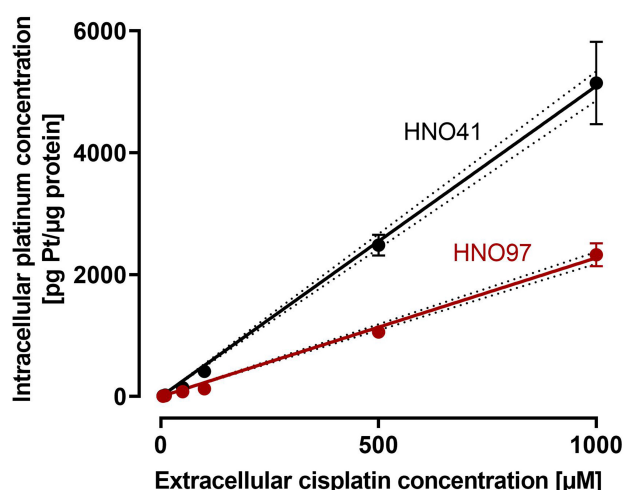


Figure 2. Cisplatin uptake characteristics in HNO41 (black) and HNO97 (dark red). Cells were exposed to cisplatin for 2.5 h, and total intracellular platinum concentrations were quantified. Data represent mean $\pm$ SD of four independent biological replicates. Linear regression lines with 95%CI margins (dotted lines) are depicted. SD: Standard deviation.

damage responses in HNO97 cells 4 h after the 2.5-h cisplatin treatment, western blot analysis was performed. Cleaved caspase 3 (an indicator of caspase 3 activation) and cleaved PARP (a downstream target of caspase 3) were assessed. Both cleaved caspase 3 (~17 kDa) and cleaved PARP (~100 kDa) were readily detectable in cisplatin-treated HNO41 cells but absent in cisplatin-treated HNO97 cells [Figure 3C]. Notably, total PARP levels were unchanged across different cells and treatment conditions. GAPDH served as the loading control and confirmed consistent protein loading.

Drug treatment-related changes in gene expression

HNO41 and HNO97 cells were treated with cisplatin, resulting in comparable total intracellular platinum concentrations. After drug exposure, the cells were placed in drug-free medium for 4 h and then subjected to PCR array-based transcriptomic analysis targeting key DNA damage and cell cycle arrest genes. In HNO41

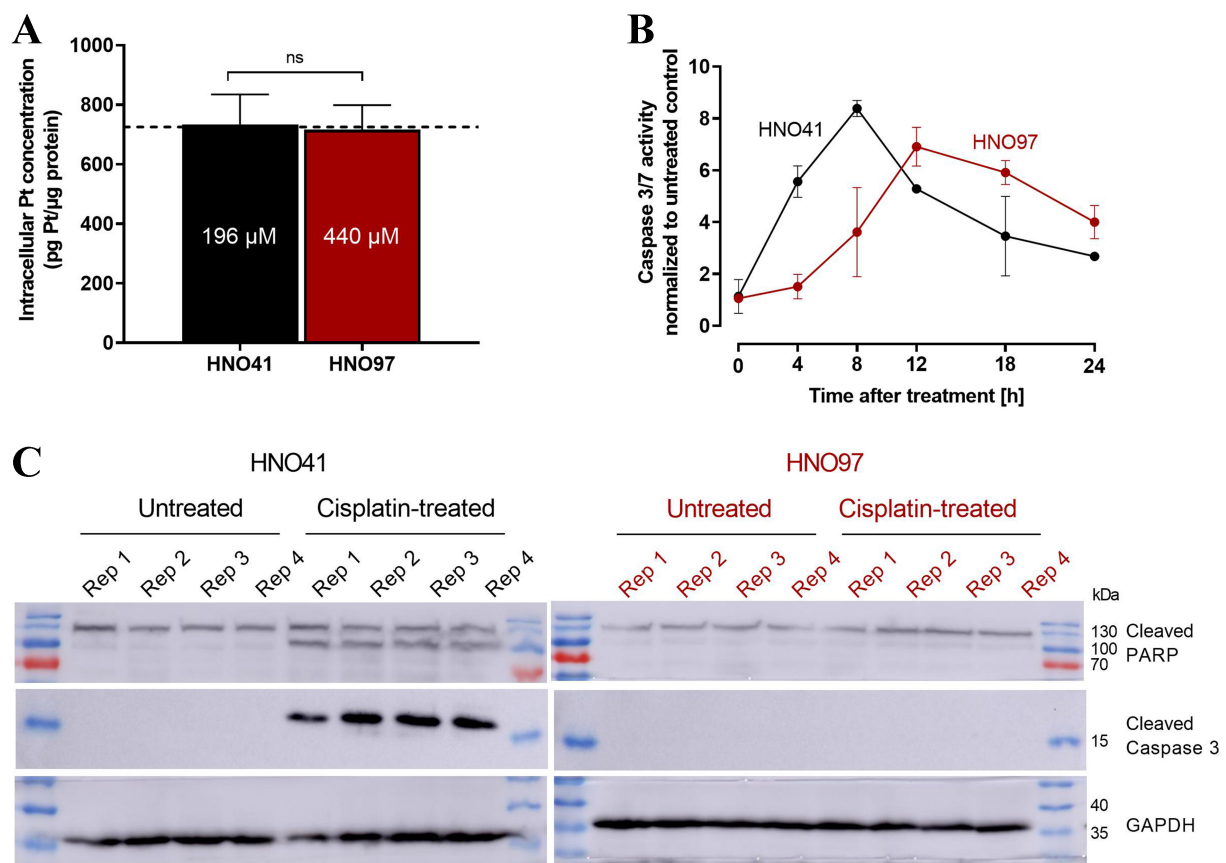


Figure 3. (A) Validation of similar total intracellular platinum concentrations (725 pg Pt/ μg protein; dotted line) after exposure of HNO41 (black) to 196 μM cisplatin and HNO97 (dark red) to 440 μM cisplatin. Data represent mean $\pm$ SD of four independent biological replicates. Statistical significance was evaluated by the non-parametric Mann-Whitney test; ns: not significant; (B) Caspase 3/7 activity in HNO41 (black) and HNO97 (dark red) over time after 2.5 h of cisplatin treatment (HNO41: 196 μM ; HNO97: 440 μM), yielding comparable intracellular platinum concentrations. Data represent mean $\pm$ SD of four independent biological replicates; (C) Western blot analysis of cleaved caspase 3 and cleaved PARP in HNO41 (black, left) and HNO97 (dark red, right), 4 h after 2.5 h of cisplatin treatment (HNO41: 196 μM ; HNO97: 440 μM) or in untreated controls (four independent biological replicates each). Twenty μg of protein lysates were resolved on a 12% gel and transferred to a PVDF membrane by wet transfer. Membranes were probed with antibodies against cleaved caspase 3 and PARP. GAPDH was used as a loading control. SD: Standard deviation.

cells, numerous genes showed substantial changes in expression [Figure 4 and Supplementary Table 1]. For instance, *GADD45G* was induced 25-fold ($2^{4.67}$), *CDKN1A* nearly 14-fold ($2^{3.78}$), *GADD45A* nearly 13-fold ($2^{3.68}$), and *PPP1R15A* 8.6-fold ($2^{3.1}$). By contrast, HNO97 cells exhibited weaker induction of these genes [Figure 5 and Supplementary Table 2]: *GADD45G* increased only 16-fold (2^4), *CDKN1A* 3-fold ($2^{1.6}$), *GADD45A* 8-fold (2^3), and *PPP1R15A* 4.75-fold ($2^{2.2}$). In both cell lines, several genes were suppressed by approximately 50% [Figures 4 and 5, Supplementary Tables 1 and 2].

Baseline expression levels

Besides treatment-related changes, baseline expression levels were also evaluated. Compared with HNO41, HNO97 cells exhibited higher mRNA expression of several genes related to DNA damage response and cell cycle arrest. For instance, *CDKN1A* expression was 7-fold higher ($2^{2.77}$), *TP53* and *OGG1* were each 6-fold higher ($2^{2.6}$), and *FANCD2* was 5.7-fold higher ($2^{2.5}$) [Figure 6 and Supplementary Table 3]. The complete expression profiles of both cell lines, under baseline and drug-treated conditions, are further presented in a heat map [Figure 7].

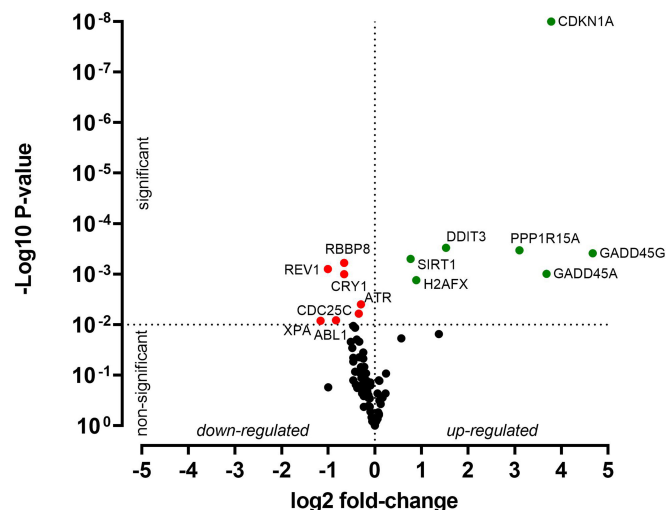


Figure 4. Cisplatin-induced gene expression changes in HNO41 cells. Cells (three independent biological replicates) were exposed to 196 μ M cisplatin for 2.5 h, transferred to drug-free medium for 4 h, and then analyzed by PCR. Significantly induced genes are highlighted in green, significantly suppressed genes in red, and non-significantly altered genes ($P > 0.01$) in black. PCR: Polymerase chain reaction.

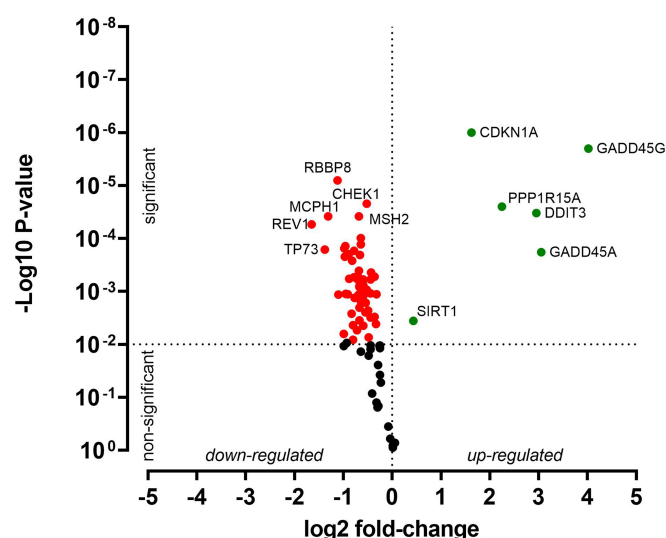


Figure 5. Cisplatin-induced gene expression changes in HNO97 cells. Cells (three independent biological replicates) were exposed to 440 μ M cisplatin for 2.5 h, transferred to drug-free medium for 4 h, and then analyzed by PCR. Significantly induced genes are highlighted in green, significantly suppressed genes in red, and non-significantly altered genes ($P > 0.01$) in black. PCR: Polymerase chain reaction.

Clinical significance of identified candidate genes

To evaluate the clinical relevance of the identified genes for the survival of HNSCC patients, a TCGA-based analysis was performed. The dataset comprised 442 human papillomavirus-negative HNSCC patients (127 females, 315 males; mean age at diagnosis: 61.5 years) with tumors located in the hypopharynx ($n = 6$), larynx ($n = 112$), oral cavity ($n = 292$), or oropharynx ($n = 32$). The documented median recurrence-free survival was 17.6 months. Normalized gene expression values were dichotomized at the cohort median to define “high” and “low” expression groups. High expression of *GADD45G* (adj. $P = 0.0725$), *PPP1R15A* (adj. $P = 0.0725$), or *FANCD2* (adj. $P = 0.0625$) was associated with shorter recurrence-free survival [Figure 8]. In addition, the combined mean expression of genes strongly induced in the cisplatin-resistant HNO97 cell line (*CDKN1A*, *GADD45A*, *GADD45G*, and *PPP1R15A*) and genes highly expressed at baseline in HNO97 (*FANCD2*, *OGG1*, *TP53*) was also associated with poor recurrence-free survival (adj. $P = 0.0625$, adj. $P =$

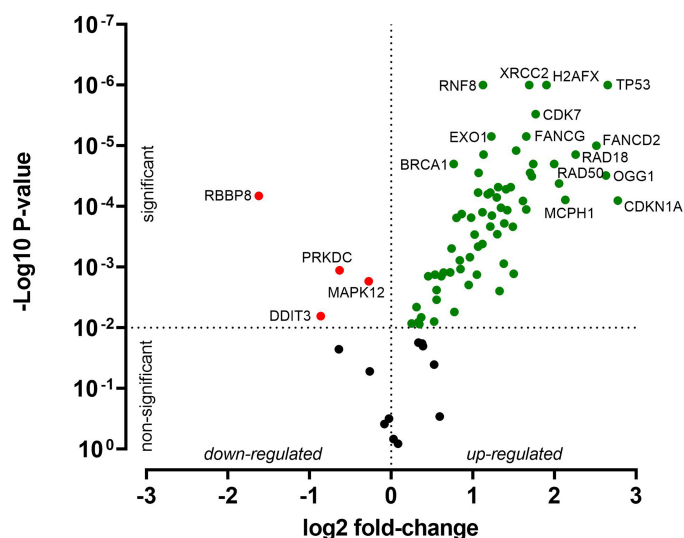


Figure 6. Relative baseline gene expression differences between HNO97 cells (three independent biological replicates) and HNO41 cells (three independent biological replicates). Genes significantly upregulated in HNO97 compared with HNO41 are highlighted in green, significantly downregulated genes are highlighted in red, and genes with no significant difference ($P > 0.01$) are shown in black.

0.093) [Figure 8]. In multivariate survival analysis, *FANCD2* remained significantly associated with poor recurrence-free survival [Table 1]. Importantly, in the subgroup of patients treated with platinum-based chemotherapy, high *FANCD2* expression ($P = 0.011$) was again associated with unfavorable recurrence-free survival [Table 2], whereas its impact was minor among patients who did not receive platinum-based chemotherapy [Table 2 and Figure 9A]. This association was further strengthened after adjusting for clinical stage and tumor site, with a significant hazard ratio of 4.0 compared to 1.4 [Figure 9B].

DISCUSSION

To the best of our knowledge, this is the first study to evaluate the relative contribution of reduced drug uptake and altered molecular responses to the cisplatin resistance phenotype in HNSCC cell lines. This was achieved by comparing concentration-response relationships not only for nominal exposure concentrations but also for resulting intracellular platinum concentrations. The *in vitro* data revealed several important findings. By comparing these two approaches, we estimated that impaired drug uptake accounts for approximately 30% of the observed cisplatin resistance in the two cell lines. The observation of reduced cisplatin uptake in HNO97 cells is supported by our previous data, which showed that HNO97 cells exhibit low levels of the cisplatin uptake transporter *SCL31A1* (human copper transporter 1) and relatively high levels of the cisplatin efflux transporters *ATP7A* and *ATP7B*^[18].

Beyond reduced uptake, the remaining ~70% of cisplatin resistance appears to arise from molecular mechanisms, meaning that HNO97 cells respond differently to the drug even when intracellular platinum levels are comparable to those in the sensitive HNO41 cells. Indeed, when both cell lines were exposed to similar intracellular platinum levels (725 pg Pt/ μ g protein), caspase 3/7 activity in HNO97 cells was delayed in onset and peak compared with HNO41. At the protein level, HNO97 showed no cleavage of caspase 3 or PARP 4 h after the end of the 2.5-h treatment phase, while western blots of HNO41 cells showed strong bands of cleaved caspase 3 and cleaved PARP. At the transcriptional level, several genes involved in cell cycle arrest and DNA damage response were differently regulated. For instance, *GADD45A*, *GADD45G*, and *PPP1R15A* were significantly more strongly induced in HNO41 than in HNO97. Most notably, *CDKN1A* expression increased 14-fold in HNO41 cells but only 3-fold in HNO97 cells. However, under untreated conditions, HNO97 cells already exhibited a 7-fold higher baseline expression of this cell cycle regulator,

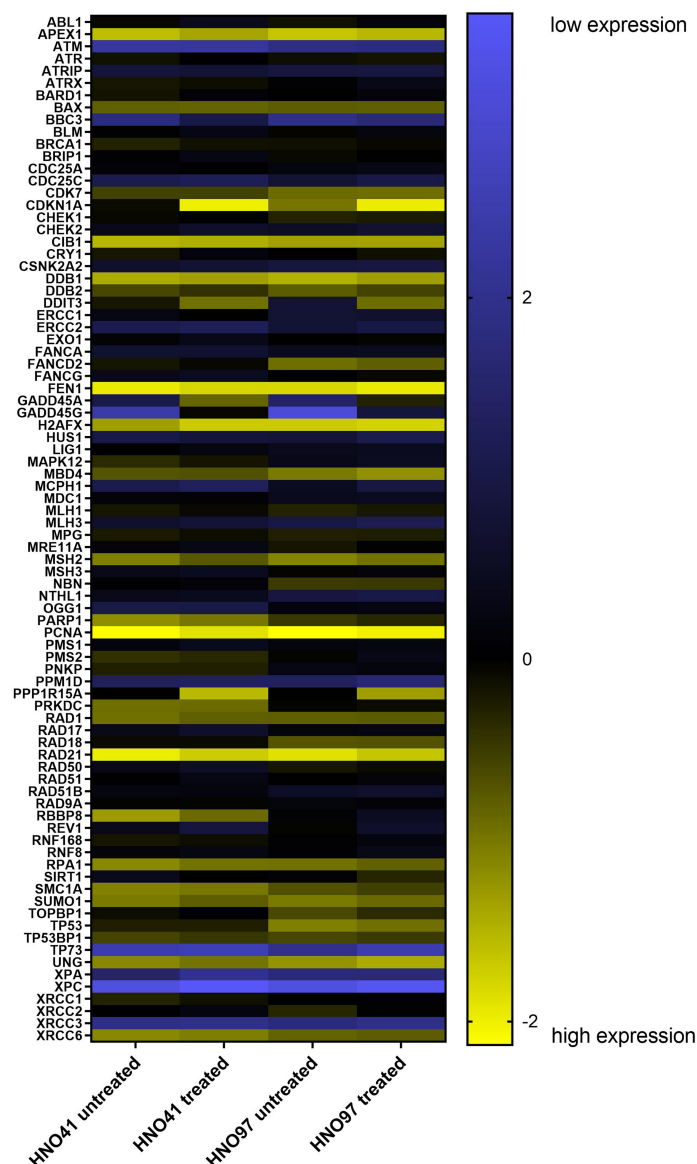


Figure 7. Heat map visualization of mRNA expression levels in untreated and treated HNO41 and HNO97 cells. Mean expression values from three independent replicates were Z-transformed and color-coded, with yellow indicating high expression and blue indicating low expression.

whose elevated expression has been associated with cisplatin resistance in bladder cancer^[22], testicular cancer^[23,24], and non-small cell lung cancer^[25]. Moreover, HNO97 cells exhibited 4- to 8-fold higher baseline mRNA expression levels of *FANCG*, *FANCD2*, *H2AFX*, and other genes involved in DNA damage response and cell cycle arrest compared with HNO41, further substantiating the molecular basis of their cisplatin resistance. This cell line has also been reported to be markedly hypomethylated in the promoter regions of DNA damage response genes, resulting in high expression of *NEIL1*, a bifunctional DNA glycosylase involved in base excision repair^[26]. Together, these findings strongly suggest that the enhanced DNA damage response capacity of HNO97 cells underlies their cisplatin resistance phenotype. To assess the clinical relevance of the identified genes in HNSCC prognosis, we performed a TCGA-based RNA-seq data set analysis. The results confirmed that the selected genes significantly correlate with recurrence. For instance, high *FANCD2* expression was associated with poor recurrence-free survival in HNSCC in both univariate and multivariate analyses. The specific importance of *FANCD2* was highlighted when the analysis was

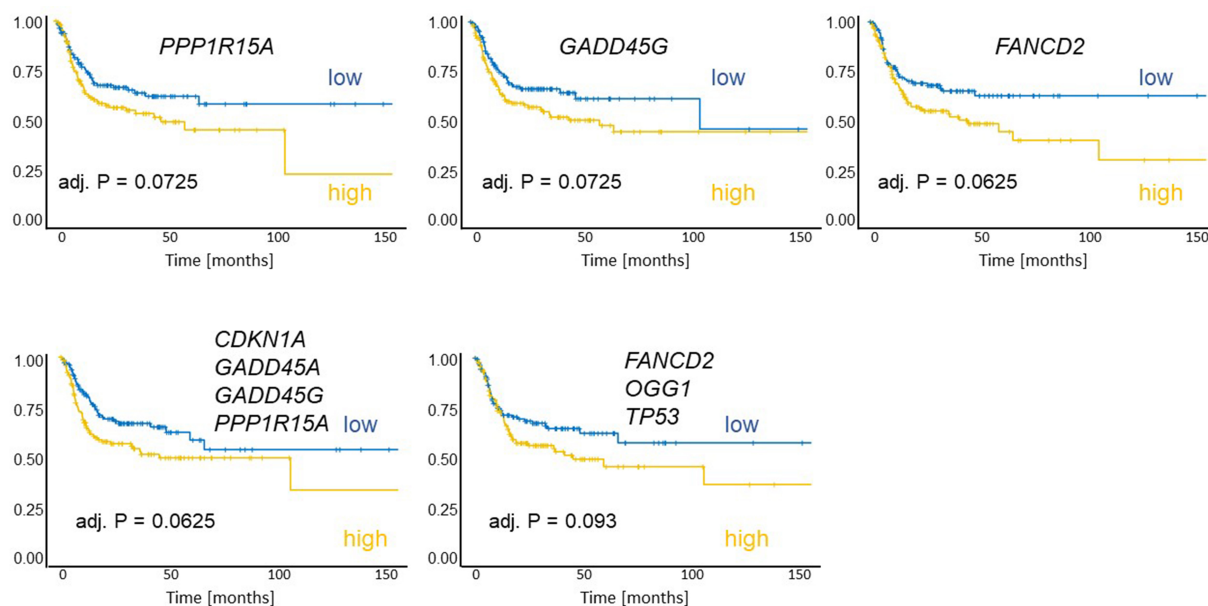


Figure 8. Kaplan-Meier plots of recurrence-free survival were generated for 292 patients. Patients were grouped according to the expression of individual genes (*PPP1R15A*, *GADD45G*, *FANCD2*), the *in vitro* treatment-induced signature (mean of: *CDKN1A*, *GADD45A*, *GADD45G*, *PPP1R15A*), and the baseline-high signature (mean of: *FANCD2*, *OGG1*, *TP53*). High expression of these genes or signatures was associated with worse outcomes. Each group comprised 146 patients.

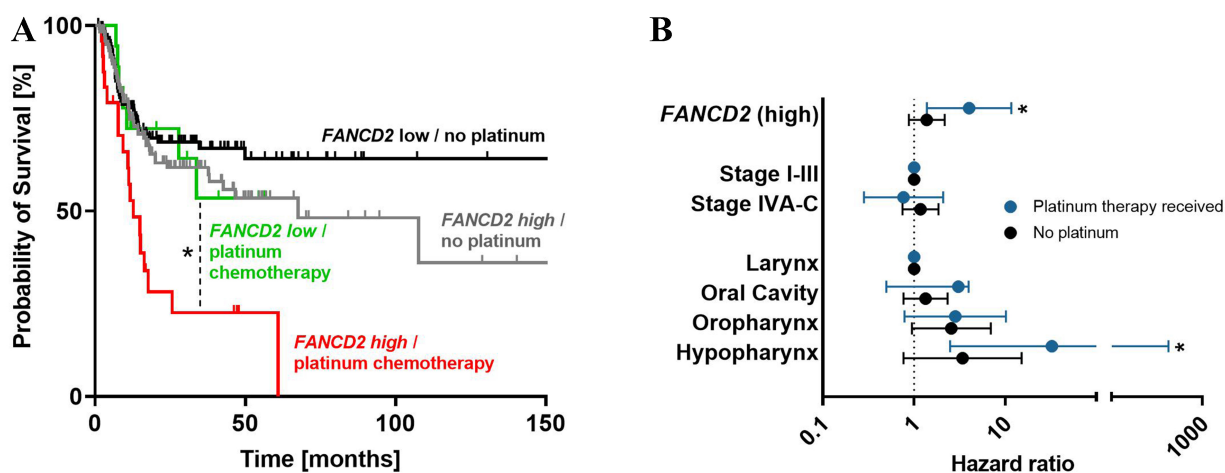


Figure 9. (A) Kaplan-Meier plots of recurrence-free survival stratified by *FANCD2* expression and adjuvant platinum-based chemotherapy indicate that high *FANCD2* expression predicts poor survival in patients treated with platinum-based chemotherapy; (B) This adverse effect remained significant after adjustment for clinical stage and tumor localization in multivariate Cox proportional hazard regression. * $P < 0.05$.

restricted to patients who received platinum-based chemotherapy. The role of *FANCD2* in cisplatin resistance is further supported by numerous previous studies^[27-30], including experimental evidence showing that reducing *FANCD2* expression (e.g., by RNA interference) or inhibiting its protein activity with small molecules sensitizes cancer cells to cisplatin^[31,32]. In summary, integrating our findings with previously published data, we conclude that altered DNA damage response plays a major role in driving the cisplatin resistance phenotype in HNSCC.

This study has several limitations. First, the HNSCC cell lines were exposed to cisplatin for only 2.5 h, and therefore, the effects of other exposure times could not be evaluated. However, a longer exposure would likely have caused substantial cytotoxic cell loss during treatment. Detached cells would have carried

Table 1. Multivariate survival analysis of recurrence-free survival using Cox proportional-hazard regression

Factor		P value	Hazard ratio	Lower 95%CI	Upper 95%CI
FANCD2	Low	-	-	-	-
	High	0.021*	1.609	1.074	2.411
	Larynx	-	-	-	-
Location	Oral Cavity	0.178	1.398	0.859	2.277
	Oropharynx	0.011*	2.638	1.254	5.552
	Hypopharynx	0.013*	4.725	1.384	16.139
Clinical stage	I-III	-	-	-	-
	IVA-C	0.286	1.245	0.833	1.861
In vitro treatment-induced signature	Low	-	-	-	-
	High	0.035*	1.515	1.029	2.229
	Larynx	-	-	-	-
Location	Oral cavity	0.352	1.253	0.779	2.014
	Oropharynx	0.014*	2.548	1.211	5.359
	Hypopharynx	0.054	3.291	0.979	11.065
Clinical stage	I-III	-	-	-	-
	IVA-C	0.203	1.296	0.869	1.933

CI: Confidence interval; P values < 0.05 are shown in bold with asterisks.

Table 2. Multivariate survival analysis of recurrence-free survival using Cox proportional-hazard regression in patients with or without platinum-based chemotherapy

Factor		P value	Hazard ratio	Lower 95%CI	Upper 95%CI
Platinum-based chemotherapy received (n = 42)					
FANCD2	Low	-	-	-	-
	High	0.011*	3.997	1.377	11.599
	Larynx	-	-	-	-
Location	Oral cavity	0.059	3.047	0.958	9.689
	Oropharynx	0.112	2.821	0.786	10.126
	Hypopharynx	0.008	32.262	2.472	421.056
Clinical stage	I-III	-	-	-	-
	IVA-C	0.602	0.766	0.282	2.084
Without platinum-based chemotherapy (n = 250)					
FANCD2	Low	-	-	-	-
	High	0.165	1.375	0.877	2.158
	Larynx	-	-	-	-
Location	Oral cavity	0.310	1.334	0.765	2.324
	Oropharynx	0.065	2.554	0.944	6.910
	Hypopharynx	0.108	3.388	0.766	14.991
Clinical stage	I-III	-	-	-	-
	IVA-C	0.493	1.173	0.744	1.849

CI: Confidence interval; P values < 0.05 are indicated in bold with asterisks.

significant amounts of platinum with them, thereby confounding the evaluation of the relationship between intracellular platinum content and anti-proliferative effects, and biasing subsequent analyses through selective enrichment of resistant cells. By contrast, 2.5 h was sufficient to ensure substantial drug uptake

while avoiding cellular damage and selection effects during treatment. After 2.5 h of drug exposure, the cells were washed and transferred to drug-free medium. Consequently, all subsequent cellular or molecular effects can only result from the platinum taken up during this period. The plasma half-life of cisplatin is about 30 min^[33]. After five half-lives, cisplatin should be cleared from plasma and the extracellular space of tumor cells by 96.875%. Thus, an *in vitro* exposure of 2.5 h (mimicking five half-lives) approximates this clinical scenario reasonably well. Second, the cisplatin concentrations used during the 2.5-h exposure for transcriptomic analysis (HNO41: 196 μ M; HNO97: 440 μ M) were relatively high. However, these supra-pharmacological concentrations were deliberately chosen. They were designed not to kill the cells during exposure but to enable quantifiable intracellular accumulation while imposing sufficient cellular stress, as reflected in gene expression alterations. Overall, the aim was to characterize the downstream responses of two unrelated cell lines when challenged with comparably high intracellular cisplatin levels. Third, anti-proliferative cisplatin effects were normalized to total intracellular platinum rather than to DNA-bound platinum, although previous comprehensive analysis had shown that DNA adduct levels are the most cytotoxic determinant across 19 different HNSCC cell lines. Martens-de Kemp *et al.* reported that cisplatin sensitivity (IC_{50} values ranging from 0.15 to 4 μ M) was not correlated with the expression of cisplatin transporters (e.g., *ATP7B*, *SLC31A1*) or DNA damage repair genes (e.g., *ERCC1*), but was highly linked to the amount of platinum bound to DNA (0.1 pmol Pt/ μ g DNA to > 1 pmol Pt/ μ g DNA)^[34]. Measuring DNA adduct levels in our experiments would therefore have provided additional insight. However, increasing evidence indicates that cisplatin targets not only DNA but also RNA, proteins, and lipids^[35-38], often resulting in only a weak correlation between DNA platination and cytotoxic efficacy^[39]. This supports the rationale for quantifying total cellular platinum instead. Fourth, only two representative but unrelated HNSCC cell lines were examined from our panel^[18]. Although baseline expression of many DNA damage repair genes was higher in the resistant HNO97 line compared to the sensitive HNO41 line, it remains unclear whether this difference is causally linked to resistance. Additional factors - such as apoptotic regulators or oxidative stress response genes not included in the PCR array - may also contribute. This limitation restricts the generalizability of our findings to the broader HNSCC population.

Despite all these weaknesses, it also presents several clear strengths. One key advantage is the quantitative assessment of the relative contribution of drug uptake to the overall resistance phenotype. While other studies have attempted similar analyses using different approaches, their methods had limitations. For instance, in one study of ovarian cancer cell lines (A2780 and a 13-fold more resistant subline), cellular platinum levels after exposure to the respective IC_{50} concentrations (3 and 40 μ M) were found to be similar, leading the authors to conclude that drug uptake was the primary factor driving cisplatin resistance^[40]. In another study, the parental ovarian cancer cell line and several cisplatin-resistant sublines were analyzed for cisplatin IC_{50} values (after 2 h exposure) and drug uptake (also after 2 h). The fold-differences for both IC_{50} values (approximately 3-4-fold higher) and cisplatin accumulation (approximately 3-4-fold lower) in resistant cells closely mirrored each other, leading to a similar conclusion that altered drug uptake was the major contributor to cisplatin resistance in these cell lines^[41]. However, these conclusions were based on measurements at only a single drug exposure concentration (uptake at IC_{50} ^[40]; uptake at 25 μ M^[41]). In contrast, our study evaluated the concentration-response relationship in two complementary ways: first, by plotting nominal drug concentrations in the medium, and second, by measuring the resulting intracellular concentrations. This dual approach provides a more accurate and robust quantification of the impact of altered drug uptake on cisplatin cytotoxicity. A second strength of our study is the detailed analysis of molecular responses at comparable total intracellular platinum concentrations, using a PCR array system that simultaneously quantifies 84 genes involved in DNA damage response and cell cycle arrest. This experimental framework can serve as a template for studies involving other drugs, cell lines, or patient-derived organoids. Finally, the clinical relevance of the identified genes was confirmed through a comprehensive analysis of TCGA data. For instance, *FANCD2* was validated in a retrospective clinical

analysis, demonstrating a significant association with poor recurrence-free survival. Importantly, the pharmacological relevance of FANCD2 for cisplatin therapy was highlighted by the observation that its expression impacted survival only in patients receiving platinum-based chemotherapy, and not in the overall HNSCC population.

In conclusion, cisplatin resistance in HNSCC seems to be largely mediated by molecular mechanisms, such as DNA damage repair and cell cycle arrest regulation, and only minimally influenced by reduced cisplatin uptake.

DECLARATIONS

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Authors' contributions

Designed the study, performed the experiments and analysis, and wrote the manuscript draft: Sandu K, Warta R, Biswas U, Zhang W, Theile D

Supervised the study, discussed the data, and made manuscript revisions: Michl P, Herold-Mende C, Weiss J

Availability of data and materials

All data can be found in the article and its [Supplementary Materials](#). Raw data are available from the corresponding author upon reasonable request.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

This study did not involve any new experiments. The HNSCC cell lines (HNO97 and HNO41) were previously established with appropriate ethics approval and patient informed consent, as reported in the original publications. Analyses of human data were performed using publicly available TCGA datasets, for which informed consent and ethical approval were obtained by the TCGA project.

Consent for publication

Not applicable.

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Supplementary Materials

[Supplementary Materials](#)

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Distinct adaptive strategies to cisplatin, vinblastine and gemcitabine in a panel of chemoresistant bladder cancer cell lines

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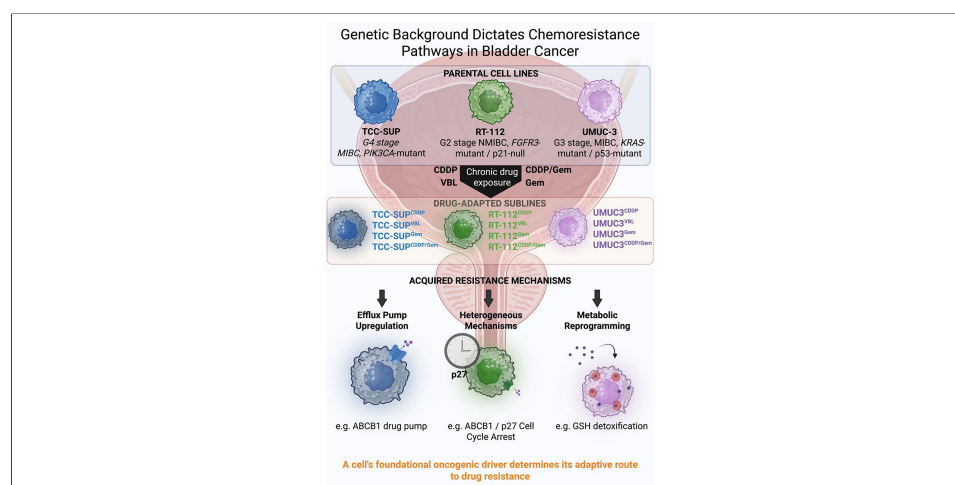
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Abstract

Aim: Urinary bladder cancer (UBC) often develops chemoresistance, reducing treatment effectiveness. This study aimed to investigate diverse molecular mechanisms underlying acquired resistance by establishing and characterizing a comprehensive panel of UBC cell lines resistant to common chemotherapeutics.

Methods: Fifteen UBC cell lines were examined: three parental lines (RT-112, TCC-SUP, UMUC-3) and twelve derived sublines adapted to cisplatin, vinblastine, or gemcitabine. Drug sensitivity was assessed using the SRB assay. Resistance mechanisms were explored via quantitative real-time PCR (targeting genes including *ABCB1*, *dCK*, *hENT1*, *ECHDC1*, *TUBB3*), Western blotting (assessing proteins such as p21, Cyclin B, and Mcl-1), and biochemical assessment of glutathione levels and redox state.

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Results: The adapted sublines exhibited distinct resistance profiles and cross-resistance patterns. Gene expression and protein analyses revealed drug- and lineage-specific alterations, involving factors such as p21, Cyclin B, and Mcl-1. Changes in glutathione metabolism were also associated with resistance. Notably, no single, universal mechanism accounted for resistance across the entire panel.

Conclusion: UBC cells develop diverse, context-dependent adaptive strategies to resist cisplatin, vinblastine, and gemcitabine. These findings highlight the complexity of chemoresistance mechanisms. The characterized cell line panel represents a valuable resource for future studies aimed at understanding and overcoming drug resistance in bladder cancer, suggesting that personalized therapeutic approaches may be necessary.

INTRODUCTION

Urinary bladder cancer (UBC) is the tenth most common malignant disease and the thirteenth leading cause of cancer-related deaths worldwide. Men are more often affected than women, ranking sixth among the most frequently diagnosed cancers in men. In 2020, an estimated 573,000 new cases of bladder cancer were diagnosed, with 213,000 deaths recorded globally^[1]. Most bladder cancers originate from the transitional (urothelial) epithelium lining the inner bladder wall, which is in direct contact with urine. This explains why tobacco smoking (responsible for half of all cases^[2]), as well as exposure to contaminated water and toxic industrial chemicals (e.g., in textiles, paints, and rubber), are the major risk factors for transitional cell carcinomas (TCCs). Because these carcinogens accumulate gradually, bladder cancer incidence is strongly age-related^[3].

Bladder cancer is currently treated with several well-established, disease-stage-dependent regimens. Non-muscle-invasive cancers are typically managed with transurethral resection followed by intravesical Bacillus Calmette-Guérin vaccine (BCG) immunotherapy, complemented with intravesical chemotherapy (e.g., doxorubicin). Muscle-invasive cancers are usually treated with either the methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) regimen or the gemcitabine and cisplatin (GC) regimen, both of which yield satisfactory results. However, a significant number of patients experience disease relapse and eventually die due to tumor metastasis. Moreover, TCCs are generally recognized as chemoresistant, and failure of first-line therapy often leads to even more refractory, life-threatening malignancies that remain difficult to treat, even with newer drugs (e.g., vinflunine)^[4,5].

Drug resistance is a well-documented phenomenon in which cancer cells gradually adapt to therapy. Resistance - whether to chemotherapy or radiotherapy - is one of the major reasons treatments fail. Some histological cancer types, including many UBCs^[6], exhibit intrinsic resistance, whereas in most cases, resistance develops in response to treatment. The mechanisms involved include increased drug efflux and/or inactivation, DNA mutations, and metabolic reprogramming that alters drug targets and enhances DNA repair^[7].

Studying the development of chemoresistance is hampered by the scarcity of paired patient samples collected before and after therapy. Since large-scale databases such as TCGA also lack such longitudinal data (if any, they contain healthy-cancerous paired samples)^[8], research in this area remains heavily dependent on *in vitro* cell line models. While many studies focus on single drug-cell line pairs, there is a notable lack of comprehensive research directly comparing how different bladder cancer cell lines adapt to multiple drugs, thus obscuring the diverse strategies underlying acquired resistance. To overcome this gap, we developed and characterized a set of isogenic cell lines with acquired resistance to cisplatin (CDDP), gemcitabine (Gem), or vinblastine (VBL). These twelve models were derived from three distinct parental lines, namely RT-112, TCC-SUP, and UMUC-3, selected from 33 urinary bladder cell lines available in the Cancer Cell Line Encyclopedia (CCLE) to represent as diverse as possible clinical and molecular features of UBC [Table 1].

Table 1. Summary of the basic features of the parental cell lines used in the current study

Feature ^[8,9]	RT-112	TCC-SUP	UMUC-3
Tumor grade	Intermediate (Grade II)	High (Grade IV)	High (Grade III)
Invasiveness	Non-muscle invasive (NMIBC)	High-grade TCC	Muscle-invasive (MIBC)
Driver mutation	<i>FGFR3-TACC3</i> (fusion)	<i>PIK3CA</i> (activating E545K)	<i>KRAS</i> (activating G12C)
TP53 (p53) status	Mutated (R248Q; S183Ter; loss-of-heterozygosity)	Mutated (E349Ter; loss-of-heterozygosity)	Mutated (F113C; copy number neutral)
CDKN1A (p21) status	Homozygous deletion (no protein)	Wild-type (inducible)	Wild-type (impaired induction)
Genomic stability	Stable (near-diploid)	Moderately unstable	Highly unstable (aneuploid)
Sensitivity to CDDP, Gem, VBL [*]	CDDP: low; Gem: moderate; VBL: high	CDDP: high; Gem: moderate; VBL: low	CDDP: high; Gem: high; VBL: moderate

^{*}Genomics of Drug Sensitivity in Cancer Database (GDSC1) accessed on 07/30/2025. TCC: Transitional cell carcinoma; CDDP: cisplatin; Gem: gemcitabine; VBL: vinblastine.

METHODS

Cell lines and culture conditions

The RT-112, TCC-SUP, and UMUC-3 cell lines were purchased from the American Type Culture Collection (ATCC; Rockville, USA). Drug-adapted sublines - RT-112^{CDDP}, RT-112^{Gem}, RT-112^{VBL}, RT-112^{CDDP/Gem}, TCC-SUP^{CDDP}, TCC-SUP^{Gem}, TCC-SUP^{VBL}, and TCC-SUP^{CDDP/Gem} - were established by continuous exposure to stepwise-increasing concentrations of the respective cytostatics and are derived from the Resistant Cancer Cell Line (RCCL) collection^[10]. The UMUC-3^{CDDP}, UMUC-3^{Gem}, UMUC-3^{VBL}, and UMUC-3^{CDDP/Gem} cell lines were established at the Hirsfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences (HIIET, PAS), Wrocław, Poland, using a similar approach.

All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Scotland) supplemented with 10% (v/v) fetal bovine serum (FBS; GE Healthcare HyClone, Logan, USA), 2 mM L-glutamine (Sigma-Aldrich, Poznań, Poland), and antibiotics - 100 µg/mL streptomycin (Polfa Tarchomin, Warsaw, Poland) and 100 U/mL penicillin (Sigma-Aldrich, Poznań, Poland). Drug-resistant sublines were cultured in media containing the corresponding cytostatics: 8.3 µM cisplatin for UMUC-3^{CDDP} and UMUC-3^{CDDP/Gem}, and 3.32 µM for RT-112^{CDDP} and TCC-SUP^{CDDP}; 5 nM vinblastine for UMUC-3^{VBL} and 20 nM for RT-112^{VBL} and TCC-SUP^{VBL}; 500 nM gemcitabine for UMUC-3^{Gem} and UMUC-3^{CDDP/Gem}, and 75 nM for RT-112^{Gem}, RT-112^{CDDP/Gem}, TCC-SUP^{Gem}, and TCC-SUP^{CDDP/Gem}. One passage before experiments, cells were allowed to grow in a drug-free culture medium.

Mycoplasma contamination was tested using the Venor GeM Classic kit (Minerva Biolabs, Berlin, Germany), with negative results in all cases. Cells were cultured in a humidified atmosphere at 37 °C with 5% (v/v) CO₂ and passaged twice weekly using ethylenediaminetetraacetic acid (EDTA)-trypsin solution (pH 8; HIIET, Wrocław, Poland) as the detachment agent.

Drugs

Bortezomib, combretastatin A-4, and vinflunine tartrate were purchased from Selleckchem (Munich, Germany) and stored at 80 °C as 50 mM solutions in dimethyl sulfoxide (DMSO; Avantor Performance Materials, Gliwice, Poland). Camptothecin, colchicine, gemcitabine hydrochloride, imatinib, trichostatin A, vinblastine sulfate, and vinorelbine tartrate were purchased from SigmaAldrich (Poznań, Poland) and stored under the same conditions. Cisplatin was purchased from Ebewe (Unterach am Attersee, Austria) and was maintained as a ready-to-use stock solution (1 mg/mL) stored at room temperature. Doxorubicin hydrochloride was purchased from Accord Healthcare Polska (Warsaw, Poland) and stored at +4 °C as a

ready-to-use stock solution (2 mg/mL). Paclitaxel was obtained from Cytoskeleton Inc. (Denver, USA) and stored at 80 °C as a 2 mM solution in DMSO.

Antiproliferative activity assessment by sulforhodamine B assay

Cells were seeded into 384-well plates (Greiner BioOne, Kremsmünster, Austria) at a density of 1×10^3 cells/well. After overnight attachment, cells were exposed to test compounds at various concentrations (at least eight concentrations). After 72 h, the sulforhodamine B (SRB) assay was performed according to Skehan *et al.*, with minor modifications^[11]. Briefly, 50 µL of culture medium was replaced with 30 µL/well of 20% (w/v) trichloroacetic acid (Avantor Performance Materials, Gliwice, Poland). After 1 h of incubation, plates were washed several times with tap water, followed by staining with 20 µL of 0.1% (w/v) SRB (Sigma-Aldrich, Poznań, Poland) in 1% (v/v) acetic acid (Avantor Performance Materials, Gliwice, Poland). After 30 min incubation at room temperature, unbound dye was removed with 1% (v/v) acetic acid, and bound dye was solubilized with 70 µL of 10 mM unbuffered tris(hydroxymethyl)aminomethane (TRIS; Avantor Performance Materials, Gliwice, Poland). The procedure was carried out using a Biotek EL-406 washing station (BioTek Instruments, USA). Absorbance was measured at 540 nm using a Biotek Hybrid H4 reader (BioTek Instruments, USA). Proliferation inhibition (%Inh) was calculated using the following formula:

$$\%Inh = \left[\left(\frac{A_p - A_m}{A_k - A_m} \right) \times 100 \right] - 100$$

where A_m is the absorbance of cell-free medium (blank), A_k is the absorbance of vehicle-treated cells (control), and A_p is the absorbance of compound-treated cells. IC_{50} (95%CI) were determined using GraphPad Prism 7.03 (GraphPad Software Inc., USA) by fitting a four-parameter variable slope equation ([inhibitor] vs. response). All values were based on at least three independent experiments.

For each cell line and compound, the resistance index (RI) was calculated as the IC_{50} ratio of resistant versus parental cell lines. RI values are reported directly as positive values when resistance was observed, and as negative values calculated as $-(1/RI)$ when sensitization occurred.

Selected drug resistance-related gene expression analysis by real-time PCR

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, USA) according to the manufacturer's instructions (based on the method developed by Chomczynski and Sacchi^[12]). RNA quantity and purity were determined spectrophotometrically at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). RNA samples were enriched with RNase inhibitors (EURx, Gdansk, Poland), treated with DNase I (Thermo Fisher Scientific, Waltham, USA) to remove genomic DNA, and reverse-transcribed into cDNA using iScript reverse transcriptase (Bio-Rad, Hercules, USA).

Real-time PCR was performed using TaqMan chemistry (Thermo Fisher Scientific, Waltham, USA). For each reaction, 25 ng of cDNA was used in technical triplicate, and four independent biological repeats were performed. Amplification consisted of 40 cycles of 95 °C for 15 s and 60 °C for 1 min, conducted on a ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA). The following TaqMan probes were used: *TUBβ3* (Hs00801390_s1); *hENT1* (Hs01085706_m1); *SLC31A1* (solute carrier family 31 member 1; Hs00741015_m1); *MT2A* (metallothionein-2; Hs02379661); *ECHDC1* (Hs00929453_m1); *SAT1* (spermidine/spermine N1-acetyltransferase 1; Hs00161511_m1); *ASS1* (argininosuccinate synthetase-1; Hs01597989_g1); *ATP7A* (P-type ATPase copper-transporting ATPase 1; Hs00163707_m1); *dCK* (Hs01040726_m1); *ABCB1* (Hs00184500_m1); and housekeeping genes *ACTB* (β-actin; Hs99999903_m1); *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase; Hs99999905_m1). *GAPDH* was selected as the reference gene. Relative expression levels (fold change) were calculated using the comparative $\Delta\Delta C_t$ method with DataAssist 3.01 software (Thermo Fisher Scientific, Waltham, USA).

Determination of the protein level by Western blot

All cells were seeded in 100-mm Petri dishes (Sarstedt, Nümbrecht, Germany). After 48 h, the culture medium was removed, and cells were washed twice with phosphate-buffered saline (PBS; HIIET, PAS, Wrocław, Poland). Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Poznań, Poland) for 15 min at 4 °C. Lysates were centrifuged at $10,000 \times g$ for 15 min at 4 °C, and the supernatants were harvested. Protein concentrations were measured using the Pierce™ Coomassie Plus assay (Thermo Fisher Scientific, Warsaw, Poland), and samples were stored at -80 °C.

Equal amounts of protein (25 µg per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4%-12%, Tris-glycine) under reducing conditions and transferred onto nitrocellulose membranes (pore size 0.45 µm; Thermo Fisher Scientific, Warsaw, Poland) using a semi-dry blotting system (Cleaver Scientific, Rugby, UK). Next, membranes were washed with Tris-buffered saline (TBS; 20 mM Tris-Base, 137 mM NaCl, pH 7.6) for 5 min at room temperature and blocked with 5% skim milk in TBS containing 0.1% Tween-20 (TBST) for 60 min. After blocking, membranes were washed three times with TBST (5 min each) and incubated overnight at 4 °C with antigen-specific rabbit IgG antibodies [anti - p21 (cyclin-dependent kinase inhibitor 1) #2947S, anti - p27 (cyclin dependent kinase inhibitor 1B) #3686S, anti - β III-tubulin #5568S, anti - Mcl1 (induced myeloid leukemia cell differentiation protein) #94296S, anti - β -tubulin #2146S, anti - Cyclin B1 #4138S, anti - ASS1 (argininosuccinate synthetase 1) #70720S, anti - MDR1/ABCB1 (multidrug resistance protein 1/ATP binding cassette subfamily B member 1) #13342S, anti - KIF11 (kinesin family member 11) #14404; all Cell Signaling Technology, Warsaw, Poland] diluted 1:1,000 in 5% bovine serum albumin (BSA) in TBST. The next day, membranes were washed three times with TBST and incubated for 1 h at room temperature with goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase (HRP; Sigma-Aldrich, Warsaw, Poland) diluted 1:1,000 in 5% BSA/TBST. After an hour incubation, membranes were washed with TBST three times for 5 min. Chemiluminescence was detected using the West Pico substrate (Thermo Fisher Scientific, Warsaw, Poland), and bands were visualized on a GelLogic 1500 imaging system (Carestream, Rochester, NY, USA).

After visualization, membranes were washed three times (10 min each) with 10 mM PBS containing 0.05% Tween (PBST, pH 7.4) and incubated for 1 h at room temperature with mouse anti- β -actin IgG antibodies (#MA5-15739; Invitrogen, Warsaw, Poland) diluted 1:4,000 in 0.5% skim milk in PBST. Following three washes with PBST, membranes were incubated for 1 h with HRP-conjugated rabbit anti-mouse IgG antibodies (#M7023, Sigma-Aldrich, Poznań, Poland) diluted 1:2,000 in 0.5% skim milk in PBST. After washing (three times, 5 min each), signals were detected as described above. Data were analyzed using ImageJ 1.8.0 and GraphPad Prism 7.03.

Intracellular glutathione level assessment

Cells were seeded in 24-well plates (Greiner BioOne, Kremsmünster, Austria) at a density of 2×10^3 cells/well. After 24 h, glutathione levels were measured using the GSH/GSSG-Glo™ assay (Promega GbhH, Walldorf, Germany) according to the manufacturer's protocol, enabling determination of both total and reduced glutathione. In parallel, experiments were conducted with cells pretreated for 24 h with 100 µM buthionine sulfoximine (BSO), an inhibitor of γ -glutamylcysteine synthetase^[13]. Results were analyzed using GraphPad Prism 7.03.

Statistical analysis

All experiments were performed at least in triplicate. Data were analyzed utilizing the appropriate statistical methods and software as specified under each section. Normality and variance were assessed with the Shapiro-Wilk and Brown-Forsythe tests, respectively. One-way ANOVA followed by appropriate post-hoc

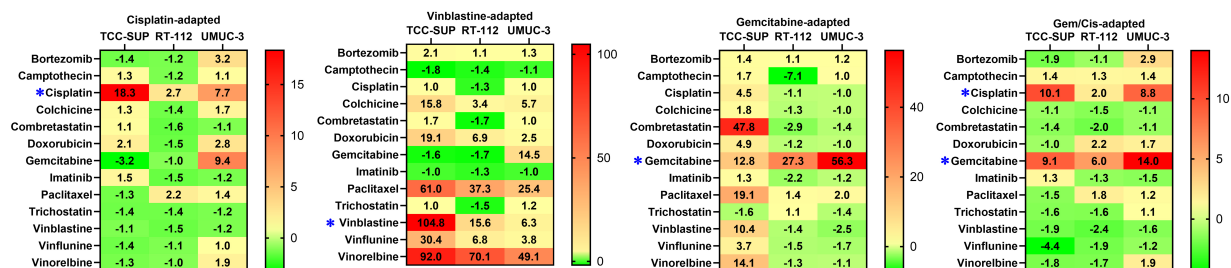


Figure 1. Drug RIs for all drug-adapted cell lines. * indicates the drug(s) used to generate each drug-adapted subline. RIs: Resistance indices.

tests was applied where applicable. Details of the statistical tests used are provided in the corresponding tables and figures.

All figures were prepared in GraphPad Prism 7.03 software.

RESULTS

UBC cell lines exhibit differential drug-resistance profiles

A set of thirteen anticancer drugs was used to determine cell line sensitivity [Table 2]. The set comprised compounds exhibiting diverse mechanisms of action and molecular targets. Minor discrepancies in drug sensitivity were observed for parental cell lines, with the ratio between the most and the least sensitive cell line exceeding 5, recorded only for camptothecin and cisplatin. However, drug-adapted cell lines showed significant differences in resistance profiles [Figure 1]. Cisplatin exposure resulted in resistance indices (RI^{CDDP}) of 18.3, 2.7, and 7.7 for TCC-SUP^{CDDP}, RT-112^{CDDP}, and UMUC-3^{CDDP}, respectively. Only minor cross-resistance was observed with doxorubicin (Dox) in UMUC-3^{CDDP} ($RI^{Dox} = 2.7$) and TCC-SUP^{CDDP} ($RI^{Dox} = 2.1$), and with bortezomib (BZT) in UMUC-3^{CDDP} ($RI^{BZT} = 3.2$). Additionally, UMUC-3^{CDDP} exhibited moderate resistance to gemcitabine ($RI^{Gem} = 9.4$), whereas TCC-SUP^{CDDP} exhibited slightly increased sensitivity to this antimetabolite ($RI^{Gem} = 0.31$). Cells exposed to vinblastine developed strong resistance, with RI^{VBL} values of 104.8, 15.6, and 6.3 for TCC-SUP^{VBL}, RT-112^{VBL}, and UMUC-3^{VBL}, respectively. Additionally, strong cross-resistance, particularly in TCC-SUP^{VBL}, was observed against other antimitotic agents (colchicine, paclitaxel, vinflunine, and vinorelbine). Interestingly, no cross-resistance was detected with combretastatin A4, another antimitotic agent. All vinblastine-resistant sublines also developed moderate to strong cross-resistance to doxorubicin (maximum $RI^{Dox} = 19.0$ in TCC-SUP^{VBL}). In addition, UMUC-3^{VBL} developed strong cross-resistance to gemcitabine ($RI^{Gem} = 14.5$). Notably, gemcitabine-treated sublines displayed high resistance, with RI^{Gem} values of 12.8, 27.3, and 56.3 for TCC-SUP^{Gem}, RT-112^{Gem}, and UMUC-3^{Gem}, respectively. TCC-SUP^{Gem} additionally developed slight cross-resistance to cisplatin ($RI^{CDDP} = 4.5$) and doxorubicin ($RI^{Dox} = 4.9$), but strong cross-resistance to combretastatin A4 (CA4; $RI^{CA4} = 47.8$), paclitaxel (PTX; $RI^{PTX} = 19.1$), vinblastine ($RI^{VBL} = 10.4$), and vinorelbine (VRL; $RI^{VRL} = 14.1$). Such extensive cross-resistance was not observed in RT-112^{Gem} or UMUC-3^{Gem}. RT-112^{Gem} showed significantly increased sensitivity to camptothecin (CPT; $RI^{CPT} = -7.1$). This phenomenon was not observed in TCC-SUP^{CDDP/Gem} ($RI^{CDDP} = 10.1$, $RI^{Gem} = 9.1$), for which even a slight increase in sensitivity toward vinflunine (VFL) was recorded ($RI^{VFL} = -4.4$). RT-112^{CDDP/Gem} developed only slight resistance to cisplatin ($RI^{CDDP} = 2.0$) and moderate resistance to gemcitabine ($RI^{Gem} = 6.0$), along with slight cross-resistance to doxorubicin ($RI^{Dox} = 2.2$). UMUC-3^{CDDP/Gem} developed moderate resistance to cisplatin ($RI^{CDDP} = 8.8$) and gemcitabine ($RI^{Gem} = 6.0$), with slight cross-resistance only to bortezomib ($RI^{BZT} = 2.8$).

Overall, drug-resistance profiling across fifteen cell lines revealed significant discrepancies among sublines derived from different parental cell lines. In some cases, extended cross-resistance emerged (e.g., TCC-SUP^{VBL}), whereas in others, sensitivity to secondary drugs remained almost unchanged (e.g.,

Table 2. Drug-resistance profiles of UBC cell lines after 72-h exposure to compounds at various concentrations, measured by SRB assay

Compound	IC ₅₀ (95%CI)				
	Parental	CDDP	VBL	Gem	CDDP/Gem
TCC-SUP					
Bortezomib (nM)	5.7 (5.2-6.2)	4.2 (3.8-4.6)	11.8 (11.2-12.5)	7.7 (7.4-8.1)	3.0 (2.7-3.1)
Camptothecin (nM)	43.5 (34.9-53.5)	55.3 (35.1-81.3)	24.2 (22.1-26.4)	71.8 (46.1-103.5)	59.8 (44.5-78.2)
Cisplatin (μM)	1.5 (1.2-1.8)	27.5 (24.4-31.0)	1.5 (1.3-1.7)	6.7 (6.0-7.4)	15.1 (13.4-17.4)
Colchicine (nM)	8.8 (8.1-9.6)	11.0 (9.7-12.6)	139.1 (100.3-192.6)	15.9 (13.1-19.5)	7.8 (6.9-8.6)
Combretastatin A4 (nM)	2.3 (2.1-2.6)	2.6 (2.3-2.9)	3.8 (3.2-4.6)	110.0 (99.8-123.6)	1.6 (1.4-1.9)
Doxorubicin (nM)	69.6 (52.8-89.5)	144.7 (109.4-185.6)	1,326 (1,155-1,517)	341.3 (274.9-418.3)	68.2 (49.1-90.1)
Gemcitabine (nM)	10.4 (3.4-20.6)	3.2 (1.5-4.9)	6.6 (5.8-7.5)	133.2 (73.2-205.1)	95.1 (80.4-111.5)
Imatinib (μM)	13.1 (12.3-13.9)	19.4 (18.2-20.6)	12.7 (11.8-13.7)	17.3 (16.7-17.9)	16.5 (15.4-17.7)
Paclitaxel (nM)	1.6 (1.4-1.8)	1.2 (1.1-1.4)	97.6 (89.6-106.0)	30.6 (27.6-33.9)	1.1 (1.0-1.3)
Trichostatin A (nM)	140.6 (124.5-159.4)	101.9 (90.7-114.2)	144.3 (123.5-166.3)	90.6 (60.5-120.5)	89.5 (69.4-108.0)
Vinblastine (nM)	0.25 (0.21-0.29)	0.22 (0.19-0.26)	26.2 (23.2-29.6)	2.6 (2.2-3.2)	0.13 (0.11-0.15)
Vinflunine (nM)	23.7 (20.6-27.0)	16.9 (14.1-20.1)	720.4 (636.8-820.0)	86.7 (76.2-98.4)	5.5 (4.1-7.0)
Vinorelbine (nM)	0.75 (0.67-0.84)	0.60 (0.50-0.71)	69.0 (57.7-82.0)	10.6 (8.8-12.7)	0.42 (0.36-0.49)
RT-112					
Bortezomib (nM)	5.7 (5.4-6.00)	4.8 (4.3-5.3)	6.0 (5.6-6.6)	6.4 (5.9-7.2)	5.3 (4.5-6.3)
Camptothecin (nM)	123.6 (83.5-172.3)	106.8 (72.3-147.6)	86.4 (58.0-122.2)	17.6 (7.7-30.5)	162.6 (101.3-238.3)
Cisplatin (μM)	9.1 (7.8-10.4)	24.2 (20.8-28.1)	7.3 (5.8-9.1)	8.3 (6.0-11.1)	17.9 (15.5-20.8)
Colchicine (nM)	21.2 (19.4-23.3)	15.4 (14.4-16.5)	72.4 (66.5-78.7)	16.7 (15.9-17.7)	14.0 (12.8-15.5)
Combretastatin A4 (nM)	7.7 (6.6-9.0)	4.9 (4.5-5.30)	4.6 (4.2-5.0)	2.7 (2.5-2.9)	3.9 (3.4-4.5)
Doxorubicin (nM)	74.6 (56.4-96.3)	50.7 (38.5-64.9)	513.7 (387.0-675.0)	60.6 (44.8-80.0)	166.0 (140.1-195.2)
Gemcitabine (nM)	11.9 (7.7-17.4)	11.6 (3.1-19.4)	7.1 (3.9-11.1)	325.0 (254.5-413.5)	70.9 (44.8-103.5)
Imatinib (μM)	13.5 (12.1-14.8)	8.9 (6.8-10.8)	10.8 (9.9-11.9)	6.2 (5.7-7.1)	10.6 (9.2-12.3)
Paclitaxel (nM)	1.2 (0.9-1.6)	2.6 (2.3-3.0)	44.8 (39.6-50.9)	1.7 (1.6-1.8)	2.1 (1.8-2.3)
Trichostatin A (nM)	112.4 (104.4-121.1)	82.6 (68.2-98.0)	76.3 (64.2-89.4)	118.8 (108.5-129.6)	70.0 (58.3-83.2)
Vinblastine (nM)	0.50 (0.43-0.58)	0.34 (0.32-0.36)	7.8 (7.1-8.6)	0.35 (0.34-0.37)	0.21 (0.19-0.22)
Vinflunine (nM)	64.4 (57.6-71.8)	56.4 (52.5-60.6)	439.6 (384.0-506.8)	43.3 (41.1-45.6)	33.4 (28.6-38.1)
Vinorelbine (nM)	0.92 (0.80-1.06)	0.90 (0.78-1.03)	64.5 (58.4-71.8)	0.74 (0.68-0.81)	0.53 (0.41-0.66)
UMUC-3					
Bortezomib (nM)	5.4 (4.8-6.1)	17.5 (15.3-20.0)	6.8 (6.0-7.8)	6.2 (5.5-7.0)	15.4 (12.9-18.2)
Camptothecin (nM)	17.0 (14.7-19.8)	19.0 (16.8-21.4)	15.1 (11.9-18.5)	17.0 (14.6-20.0)	23.6 (20.5-27.0)
Cisplatin (μM)	2.6 (2.2-2.89)	20.0 (17.9-22.2)	2.6 (1.8-3.4)	2.5 (1.7-3.3)	22.9 (20.1-26.0)
Colchicine (nM)	9.9 (8.4-11.7)	16.7 (13.7-20.4)	56.5 (44.5-72.2)	9.5 (7.4-11.4)	9.0 (7.7-10.2)
Combretastatin A4 (nM)	1.8 (1.4-2.1)	1.6 (1.2-1.9)	1.8 (1.5-2.1)	1.3 (1.1-1.6)	1.6 (1.3-2.0)
Doxorubicin (nM)	34.8 (27.5-42.8)	95.7 (82.4-110.1)	85.9 (62.6-112.9)	33.4 (29.6-37.6)	58.5 (45.8-72.8)
Gemcitabine (nM)	16.0 (15.3-16.7)	150.5 (123.8-181.7)	232.0 (211.2-255.2)	900.7 (753.6-1,066.0)	224.0 (199.7-250.0)
Imatinib (μM)	21.0 (17.7-24.8)	17.6 (15.4-20.2)	20.5 (17.4-24.3)	18.3 (16.1-20.8)	14.2 (12.6-16.3)
Paclitaxel (nM)	1.6 (1.1-2.1)	2.2 (1.9-2.6)	40.6 (36.1-45.9)	3.2 (2.3-4.3)	1.9 (1.5-2.3)
Trichostatin A (nM)	49.6 (38.5-63.0)	42.2 (23.1-63.6)	61.7 (50.3-74.6)	36.4 (27.8-46.1)	52.3 (40.8-65.3)
Vinblastine (nM)	0.83 (0.55-1.21)	0.72 (0.61-0.86)	5.2 (4.1-6.5)	0.33 (0.27-0.41)	0.53 (0.31-0.83)
Vinflunine (nM)	55.8 (45.7-67.8)	56.7 (39.0-96.6)	210.8 (158.9-279.1)	32.9 (24.4-44.5)	45.0 (35.0-58.4)
Vinorelbine (nM)	0.86 (0.72-1.03)	1.6 (1.4-1.8)	42.2 (30.6-59.7)	0.76 (0.60-0.97)	1.6 (0.9-2.7)

Data are presented as IC₅₀ (95%CI). UBC: Urinary bladder cancer; SRB: sulforhodamine B; CDDP: cisplatin; VBL: vinblastine; Gem: gemcitabine; TCC: transitional cell carcinoma.

Table 3. Representative resistance-related genes assessed in the current study

Gene	Protein name	Related drug	Mechanism	Ref.
<i>ABCB1</i>	ATP-dependent translocase	Multiple drugs	↑, drug efflux pump	UniProt no. P08183; [14]
<i>ASS1</i>	Argininosuccinate synthase	Platinum-based drugs	↓, metabolic reprogramming	UniProt no. P00966; [15-17]
<i>ATP7A</i>	Copper-transporting ATPase 1	Platinum-based drugs	Drug sequestration/efflux	UniProt no. Q04656; [18]
<i>dCK</i>	Deoxycytidine kinase	Pyrimidine antimetabolites	Mut, ↓, prevents drug activation	UniProt no. P27707; [19]
<i>ECHDC1</i>	Ethylmalonyl-CoA decarboxylase	Gemcitabine	↑, altered metabolism	UniProt no. Q9NTX5; [20]
<i>hENT1</i>	Equilibrative nucleoside transporter 1	Gemcitabine	↓; reduced drug uptake	UniProt no. Q99808; [21]
<i>MT2A</i>	Metallothionein-2	Platinum-based drugs	↓; drug inactivation	UniProt no. P02795; [22]
<i>SAT1</i>	Diamine acetyltransferase 1	Cisplatin	↓; altered polyamine metabolism	UniProt no. P21673; [17]
<i>SLC3A1</i>	Neutral and basic amino acid transport protein	Several drugs, incl. cisplatin and doxorubicin	↑, altered nutrient transport	UniProt no. Q07837; [23]
<i>TUBβ3</i>	Tubulin βIII	Tubulin-interacting agents	↑, mut, altered drug target	UniProt no. Q13509; [24]

↑: Upregulated; ↓: downregulated, mut: mutated. ATP: Adenosine triphosphate.

RT-112^{CDDP/Gem}) or even increased (e.g., RT-112^{Gem} to camptothecin). These findings indicate that the tested cell lines developed drug resistance through diverse molecular mechanisms.

UBC cell lines exhibit differential profiles of selected drug-resistance-related genes

To address discrepancies observed in drug-resistance profiling, we evaluated the expression of several genes often associated with drug-resistant phenotypes and with cancer aggressiveness, invasiveness, and poor prognosis [Table 3]. We selected a targeted set of genes to cover a broad range of potential drug resistance mechanisms. The goal was to identify markers that could distinguish between our various resistant cell lines. While this small panel cannot capture all possible resistance mechanisms - especially across three drugs with distinct modes of action - it successfully uncovered notable differences between the models.

Parental cell lines exhibited significantly different expression profiles for most of the genes tested [Figure 2]. Statistically significant differences were observed, for example, in *ABCB1* and *TUBβ3* (with RT-112 exhibiting the lowest expression), as well as in *ATP7A*, *dCK*, *SAT1*, and *SLC3A1* (with TCC-SUP exhibiting the highest expression). Discrepancies in *ASS1* expression were especially striking: TCC-SUP and RT-112 showed 2,646- and 123-fold overexpression, respectively, compared to UMUC-3.

Drug-adapted cell lines also displayed distinct expression profiles [Figure 3]. TCC-SUP^{CDDP} (the only cisplatin-adapted line in this set) showed decreased *SAT1* expression and increased *dCK* and *TUBβ3* expression. RT-112^{CDDP} exhibited reduced *ABCB1*, *ASS1*, and *MT2A* levels and increased *ECHDC1* expression. UMUC-3^{CDDP} displayed elevated *ASS1*, *ATP7A*, *MT2A*, and *SLC3A1* as well as increased *dCK*, *ECHDC1*, and *TUBβ3*. All three vinblastine-adapted cell lines exhibited significantly increased *ABCB1* expression, with the highest increases observed in RT-112^{VBL} and TCC-SUP^{VBL} (12,433- and 2,271-fold, respectively, relative to the parental lines). Because *ABCB1* expression is four times lower in RT-112 compared to TCC-SUP, the parentalcorrected *ABCB1* increase in TCC-SUP^{VBL} is nearly equivalent to that in RT-112^{VBL}. Additionally, TCC-SUP^{VBL} (the only VBL-adapted line) exhibited decreased *hENT1* and increased

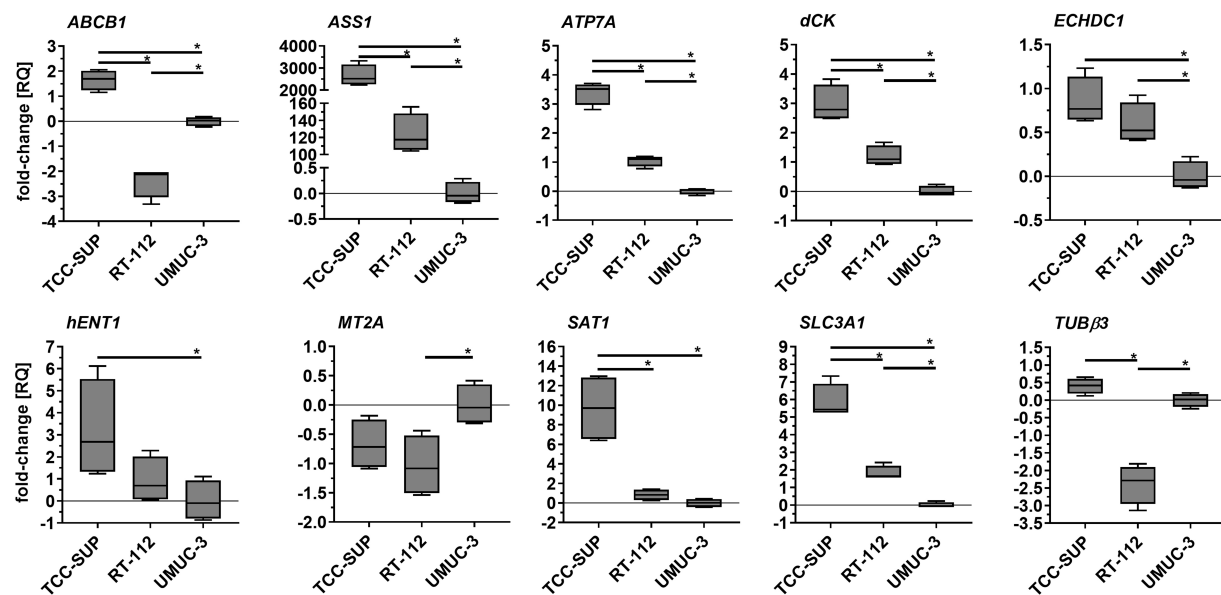


Figure 2. Comparison of selected gene expression in parental cell lines. Quantitative PCR results were normalized to *GAPDH* and expressed relative to the mean mRNA level of each gene in UMUC-3. Results ($n = 4$) are shown as box-and-whisker plots, with whiskers indicating minimum and maximum values. * $P < 0.05$, one-way ANOVA with Tukey's post-hoc test. PCR: Polymerase chain reaction; ANOVA: analysis of variance.

TUBβ3, whereas RT-112^{VBL} displayed slightly decreased *SAT1* expression. UMUC-3^{VBL} showed increased *ATP7A*, *dCK*, *ECHDC1*, *MT2A*, and *SLC3A1*. Gemcitabine-adapted lines also showed distinct patterns. TCC-SUP^{Gem} and TCC-SUP^{Gem/CDDP} (the only gemcitabine-adapted lines in this set) exhibited increased *ABCB1* and *ECHDC1*. RT-112^{Gem} and RT-112^{Gem/CDDP} displayed decreased *dCK*, *MT2A*, and *SAT1* (similar to TCC-SUP^{Gem} for *SAT1*), with additional *ASS1* downregulation in RT-112^{Gem/CDDP} and *ATP7A* upregulation in RT-112^{Gem}. UMUC-3^{Gem} and UMUC-3^{Gem/CDDP} exhibited elevated *SLC3A1* and *TUBβ3*, with variable changes in *ASS1*, *ATP7A*, *dCK*, and *ECHDC1* (both genes upregulated in the Gem/CDDP subline but downregulated or unchanged in the gemcitabine-only line).

The heterogeneous gene expression profiles observed in drug-resistant cell lines (even among lines adapted to the same drug) indicate that each line employs a different strategy to achieve a drug-resistant phenotype. This diversity is an important feature of this cell line panel when considering its use in drug resistance studies.

UBC cell lines: drug-resistant profiles associated with significant differences in selected protein levels

To further characterize the newly derived drug-adapted cell lines, we analyzed variations in selected protein levels via Western blot followed by densitometry. We focused on the protein expression of *ABCB1* and *ASS1*, as these genes exhibited the most variable transcript levels across the parental cell line panel. Additionally, we evaluated proteins linked to cell cycle progression (Cyclin B1, p21, p27), mitosis (KIF11, tubulins), and apoptosis regulation (Mcl-1). This targeted protein set allowed us to identify key differences between the cell lines, such as distinct p21/p27 expression profiles, and to further delineate significant variations in their drug resistance mechanisms, even with a limited number of proteins examined.

In the parental cell lines [Figure 4], significant differences were observed in *ASS1*, Mcl-1, Cyclin B, p21, p27, and β-tubulin, particularly between TCC-SUP and UMUC-3. Notably, p21 was not detected in RT-112 cells, consistent with the previously reported absence of functional p21 protein in this line^[25].

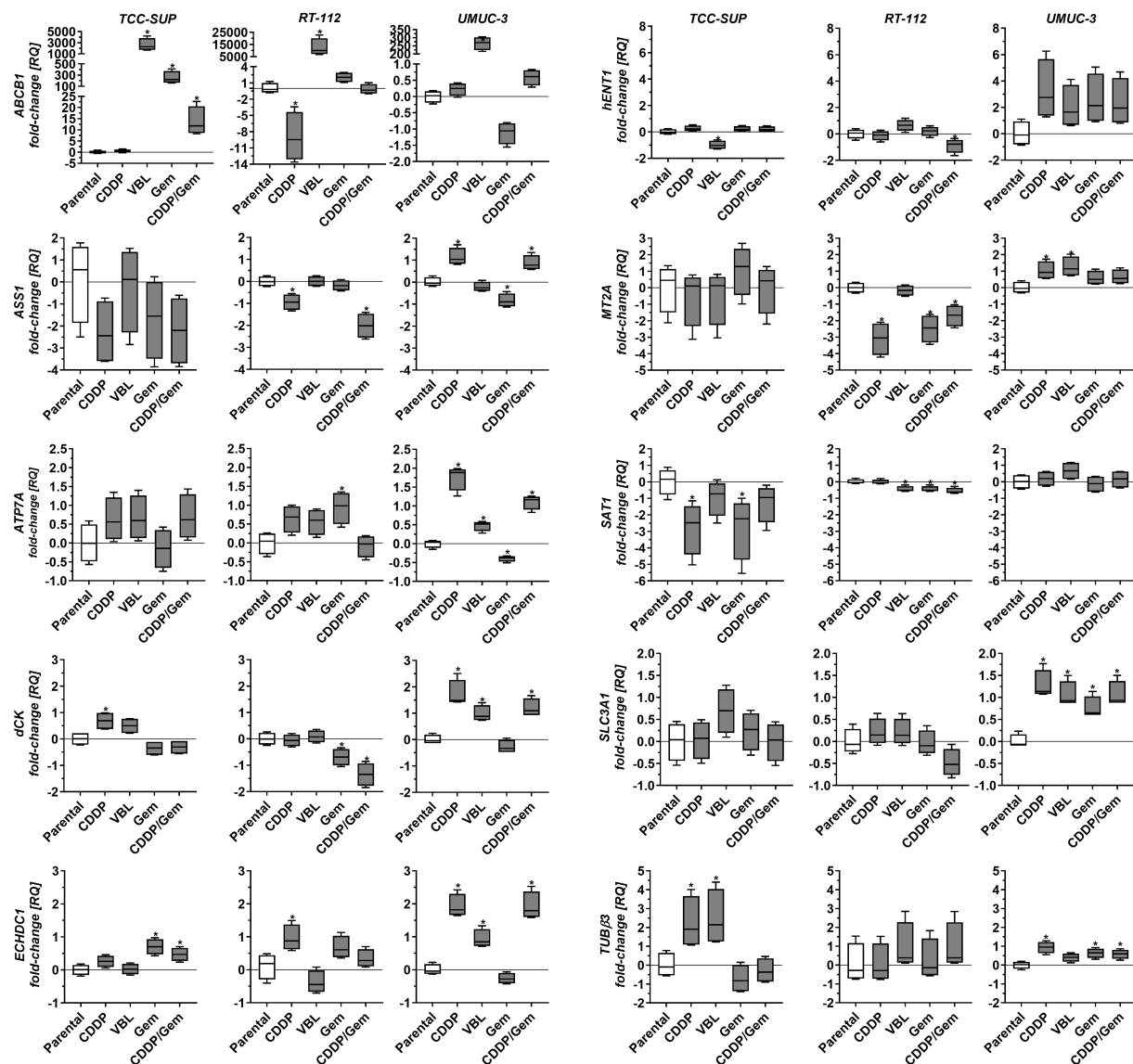


Figure 3. Comparison of selected gene expression in drug-resistant sublines relative to parental cell lines. Quantitative real-time PCR results were normalized to *GAPDH* and expressed relative to the mean mRNA level of each gene in the corresponding parental line. Results ($n = 4$) are shown as box-and-whisker plots, with whiskers indicating minimum and maximum values. * $P < 0.05$ to parental line, oneway ANOVA with Dunnett's post-hoc test. PCR: Polymerase chain reaction; ANOVA: analysis of variance.

Drug-adapted cell lines also showed significant differences in selected protein levels [Figure 5]. In cisplatin-adapted cell lines, marked changes were observed for Cyclin B, Mcl1, p21, and p27. In TCC-SUP^{CDDP} and RT-112^{CDDP} cells, Cyclin B and Mcl1 levels increased, whereas UMUC-3^{CDDP} displayed the opposite trend. p21 expression significantly decreased in TCC-SUP^{CDDP}, remained undetectable in RT-112 sublines, and showed no significant changes in UMUC-3^{CDDP}. In contrast, p27 expression increased in RT-112^{CDDP}. Importantly, TCC-SUP^{CDDP} was the only cisplatin-resistant cell line with elevated β -tubulin III expression, at levels comparable to those in TCC-SUP^{VBL}. All vinblastine-resistant cell lines showed strong expression of the ABCB1 efflux pump. Additionally, UMUC-3^{VBL} cells exhibited reduced Cyclin B and β -tubulin III, while TCC-SUP^{VBL} cells exhibited increased p27 and decreased p21. Continuous gemcitabine treatment had minimal effects on protein levels in any cell line. However, combined gemcitabine/cisplatin-adapted cell lines exhibited altered ASS1 expression (decreased in RT-112^{CDDP/Gem} and increased in UMUC-3^{CDDP/Gem}) and a slight reduction in Mcl1 in UMUC-3^{CDDP/Gem}. Notably, relative ASS1

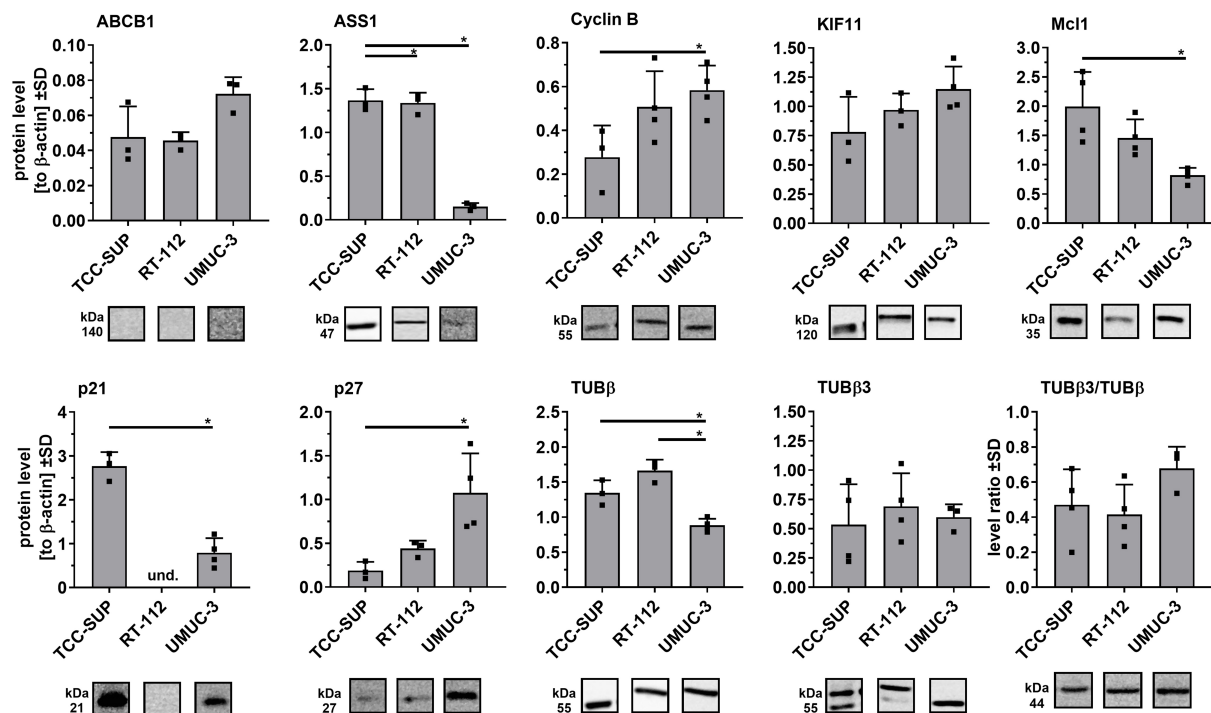


Figure 4. Comparison of selected protein levels in parental cell lines using Western blot analysis. Densitometric values for each protein ($n = 3-4$) were normalized to β -actin and presented as mean $\pm$ SD, with ■ symbols indicating raw values. $P < 0.05$, one-way ANOVA with Tukey's post-hoc test for multiple comparisons (except p21 results, analyzed by two-tailed t -test); und: undetectable. SD: Standard deviation; ANOVA: analysis of variance.

expression was consistently lower in UMUC-3 parental cells compared with TCC-SUP and RT-112. Furthermore, UMUC-3 sublines adapted to cisplatin and/or gemcitabine showed elevated KIF11, whereas RT-112^{VBL} exhibited significantly lowered levels of this kinesin.

The distinct protein expression profiles observed in drug-adapted cell lines that originated from different parental lines indicate significant discrepancies in the metabolic pathways involved in drug resistance. These observations underscore the utility of these newly developed cell lines as valuable models for studying mechanisms of drug resistance.

UBC drug-resistant cells present diverse total and reduced glutathione profiles

Since glutathione (GSH) plays a pivotal role in phase II detoxification pathways - acting as a key regulator of cellular redox status and involved in xenobiotic inactivation - we measured glutathione levels in newly established cell lines to determine whether changes in its levels contribute to drug resistance [Figure 6, left panel]. The parental TCC-SUP cell line exhibited more than twice the total glutathione level of the other parental lines, and this level did not change substantially after drug adaptation. In the RT-112 cell line series, adaptation to vinblastine resulted in a significant increase in total glutathione level (2.0-fold change), similar to the UMUC-3^{VBL} cell line (2.2-fold change). The greatest increase was observed in the UMUC-3^{CDDP/Gem} cell line (2.3-fold change). Interestingly, the parental TCC-SUP cell line exhibited the lowest GSH/GSSG ratio among all lines tested (4.5). All of its drug-adapted sublines showed a significant increase, with the highest ratios observed in TCC-SUP^{CDDP} and TCC-SUP^{CDDP/Gem} (14.6 and 12.7, respectively). This suggests that glutathione recycling to its reduced form, rather than increased synthesis, was the predominant response to xenobiotic exposure in the culture medium. In RT-112 cells (parental ratio = 10.9), adaptation to CDDP and CDDP/Gem resulted in significant increases in the GSH/GSSG ratio (15.8 and 17.8, respectively), but the fold changes (~1.5-fold) were smaller than those in TCC-SUP sublines (~3.0-fold). In contrast, the UMUC-3

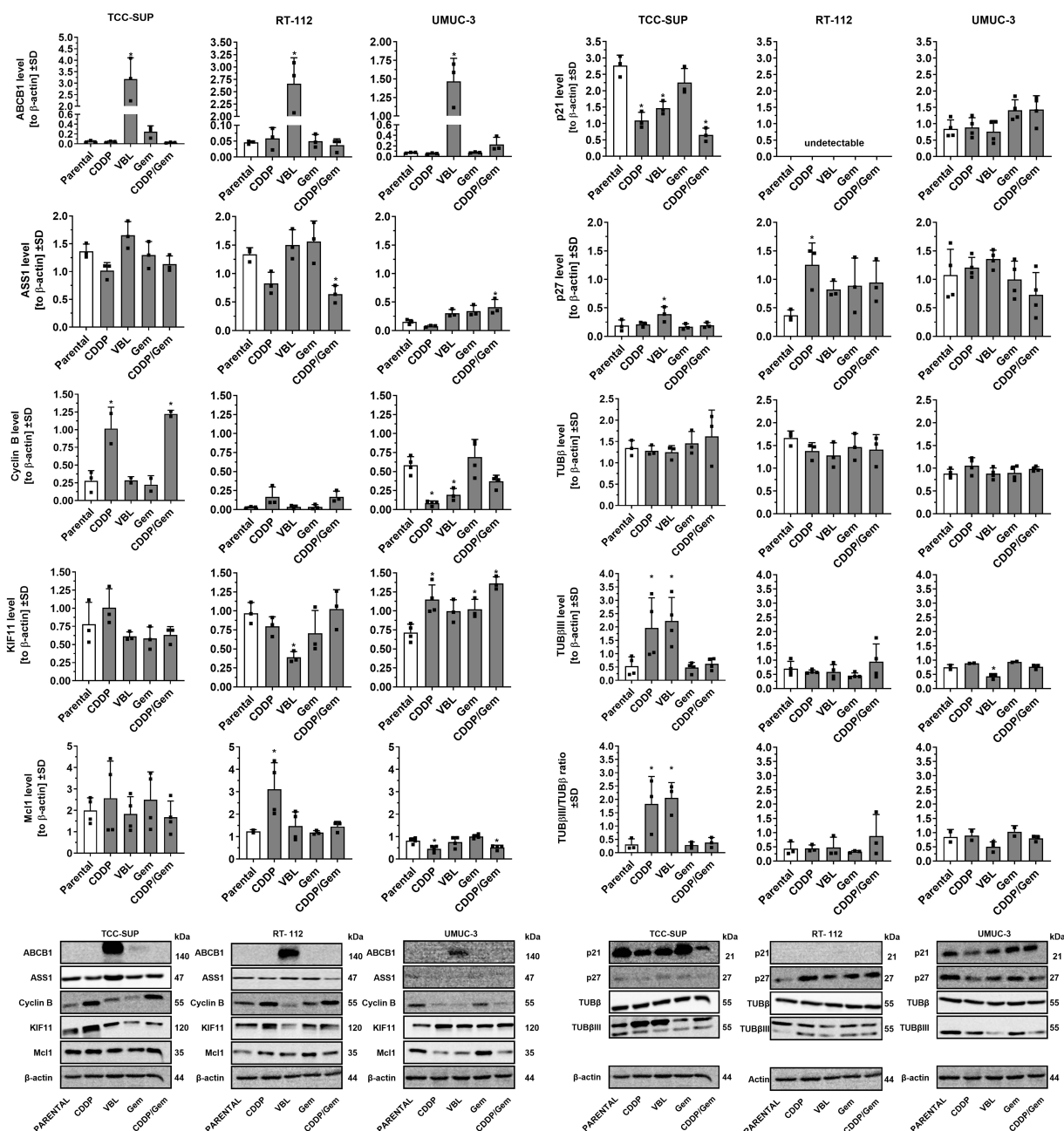


Figure 5. Comparison of selected protein levels in drug-resistant sublines versus parental cell lines. Densitometric values ($n = 3-4$) were normalized to β -actin and presented as mean $\pm$ SD, with ■ symbols indicating raw values. * $P < 0.05$, one-way ANOVA with Dunnett's post-hoc test compared to the corresponding parental cell line; und: undetectable. SD: Standard deviation; ANOVA: analysis of variance.

parental cell line showed the highest GSH/GSSG ratio (25.3), and three of its sublines (VBL, Gem, and CDDP/Gem) showed decreases, with the largest reduction observed in UMUC-3^{CDDP/Gem} (18.8). Nevertheless, UMUC-3 sublines exhibited the least substantial changes in the GSH/GSSG ratio compared to the other cell lines.

To assess cell line susceptibility to glutathione depletion, the experiments were repeated with cells treated with 100 μ M BSO for 24 h [Figure 6, right panel]. BSO treatment markedly reduced total glutathione levels in all parental lines, with the greatest decrease observed in TCC SUP (5.6-fold change). Comparable glutathione depletion was also observed in all of its sublines. Most other cell lines exhibited at least a 2-fold decrease in

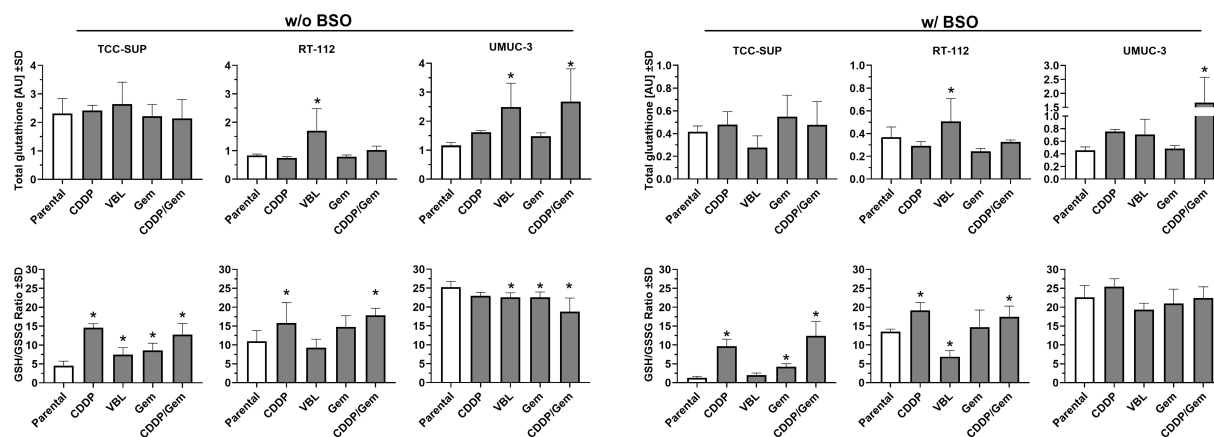


Figure 6. Comparison of total glutathione levels (upper panels) and GSH/GSSG ratio (lower panels) in drug-resistant sublines relative to their parental cell lines. The left panel shows results without BSO pre-treatment, while the right panels show results after 24 h pre-treatment with 100 μ M BSO. Data ($n = 3-4$) were analyzed according to the manufacturer's protocol and are presented as mean $\pm$ SD. $^*P < 0.05$, one-way ANOVA with Dunnett's post-hoc test, compared to the corresponding parental cell line. GSH: Glutathione; GSSG: glutathione disulfide; BSO: buthionine sulfoximine; SD: standard deviation; ANOVA: analysis of variance.

total glutathione compared to the BSO-free condition. The only exception was the UMUC-3^{CDDP/Gem} subline, which showed only a 1.6-fold reduction, suggesting higher resistance to BSO-induced depletion. The GSH/GSSG ratio did not change substantially after BSO pre-treatment; thus, parental and drug-resistant cell lines maintained comparable relationships (differences did not exceed ~ 1.5 -fold). Notable exceptions were the parental TCC-SUP cell line and its TCC-SUP^{VBL} and TCC-SUP^{CDDP/Gem} sublines, which exhibited 6.5-, 3.7- and 2.0-fold changes, respectively. These results indicate that in TCC SUP cells, glutathione depletion due to synthesis blockage is accompanied by impaired recycling of glutathione into its reduced form, potentially rendering them more susceptible to oxidative stress.

DISCUSSION

A pivotal finding of our study is that the adaptive strategies for drug resistance are profoundly influenced by the intrinsic genetic context of the parental cancer cell. The distinct resistance profiles of the TCC-SUP, UMUC-3, and RT-112 sublines can be plausibly linked to their known driver mutations (*PIK3CA*, *KRAS*, and *FGFR3*, respectively), which prime these cells for specific survival pathways when challenged with chemotherapy.

The TCC-SUP cell line, which harbors an activating *PIK3CA* (E545K) mutation, consistently developed resistance through mechanisms involving drug efflux and cell cycle deregulation. The PI3K/AKT/mTOR pathway, constitutively activated by this mutation, is a master regulator of cell growth, proliferation, and survival^[26,27]. Its chronic activation can promote resistance by broadly enhancing cell survival signals that counteract the pro-apoptotic effects of chemotherapy^[26]. This may explain why TCC-SUP sublines, particularly TCC-SUP^{VBL}, exhibited the most potent resistance, driven by upregulation of the *ABCB1* drug efflux pump, which conferred cross-resistance to other antimitotic agents recognized as *ABCB1* substrates^[28]. The PI3K pathway has also been linked to the transcriptional regulation of ABC transporter expression^[29]. Furthermore, the observed decrease in p21 and increase in Cyclin B in cisplatin- and vinblastine-adapted TCC-SUP cells suggest a strategy of overriding cell cycle checkpoints. Reduced p21 levels may enable cell cycle progression despite the presence of damaged DNA, thereby contributing to genomic instability^[30,31]. Overactivation of the PI3K/AKT pathway can phosphorylate and inactivate cell cycle inhibitors, driving proliferation even in the presence of DNA damage, a hallmark of a robust resistance phenotype^[32]. This phenotype is further supported by adaptations in glutathione metabolism; rather than increasing total

glutathione, TCC-SUP sublines enhanced glutathione recycling, as indicated by a significant increase in the GSH/GSSG ratio, thereby boosting their detoxification capacity^[33,34].

The UMUC-3 cell line, carrying an activating *KRAS* (G12C) mutation, demonstrated a preference for metabolic adaptations, particularly those involving GSH. Oncogenic *KRAS* is well-documented to rewire cellular metabolism to meet the high anabolic and redox demands of cancer cells^[35]. *KRAS* mutations drive upregulation of glycolysis and glutamine utilization, supplying precursors for proliferation but also increasing the production of reactive oxygen species (ROS) as a byproduct. To survive this self-induced oxidative stress, *KRAS*-mutant cells become highly dependent on antioxidant systems, particularly the glutathione pathway, which neutralizes ROS^[36,37]. Our finding that UMUC-3 sublines consistently modulated their GSH levels and GSH/GSSG ratio aligns with this paradigm. Their reliance on glutathione was further evidenced by adaptations such as increased total glutathione levels and overexpression of the cysteine transporter SLC3A1, a key contributor to GSH synthesis^[38,39]. For these cells, strengthening the glutathione system is a pre-existing survival mechanism that can be readily exploited to detoxify xenobiotics such as cisplatin, explaining their reliance on this pathway rather than ABCB1 upregulation.

The RT-112 cell line, characterized by an *FGFR3-TACC3* fusion, presents a more nuanced case. Activating *FGFR3* mutations are common in non-muscle-invasive bladder cancer and are typically associated with a distinct, less aggressive tumor biology^[8,40]. The *FGFR3* signaling pathway primarily influences cell proliferation and differentiation via the RAS-MAPK and PI3K-AKT axes^[41,42]. Unlike the broad, systemic effects of *KRAS* or *PIK3CA* mutations, the consequences of *FGFR3* activation are more specific. In our study, RT-112 sublines developed resistance through multiple mechanisms without a single dominant strategy as seen in TCC-SUP or UMUC-3. For instance, resistance to vinblastine involved *ABCB1* upregulation, though to a lesser extent than in TCC-SUP. In parallel, these cells also employed a distinct glutathione-based strategy, characterized by increased glutathione synthesis rather than enhanced recycling, demonstrating metabolic flexibility^[43]. Notably, studies have shown that resistance to FGFR inhibitors in *FGFR3*-driven cells can arise from the activation of bypass signaling tracks or the acquisition of new mutations, such as in *HRAS* or PI3K^[41,44]. This suggests that *FGFR3*-driven networks provide cellular plasticity, allowing for the emergence of various smaller-effect resistance mechanisms rather than reliance on a single dominant one. The increased p27 levels observed in RT-112^{CDDP} may represent one such specific adaptation, inducing cell cycle arrest that permits DNA repair and survival - a strategy not observed in the other cell lines. While p27 is a cyclin-dependent kinase inhibitor, its upregulation can act as a double-edged sword. In this context, however, p27-induced cell cycle arrest may prevent drug-induced apoptosis by providing additional time for DNA repair, ultimately enhancing cell survival^[45].

While this study provides valuable insights into the molecular underpinnings of chemoresistance in UBC, its conclusions must be considered in the context of two primary limitations: the finite genetic scope of the cellular models and the vast complexity of drug resistance mechanisms. First, our investigation was based on three distinct cell lines - TCC-SUP, RT-112, and UMUC-3 - which were deliberately selected to represent a spectrum of disease subtypes with *PIK3CA*, *FGFR3*, or *KRAS* mutations and varying degrees of genomic instability^[8,46]. This diversity was critical, as it revealed cell line-specific resistance strategies, such as the dichotomy in p21 function, which appeared to promote unchecked proliferation in TCC-SUP sublines but may act as an anti-apoptotic shield in UMUC-3^[47]. Nevertheless, it is self-evident that three cell lines cannot fully recapitulate the heterogeneity of available cancer cell lines^[8], nor the extensive intertumoral heterogeneity observed across the entire patient population^[48,49]. The unique genetic background of each line is likely a key determinant of the divergent resistance pathways we observed, underscoring the need for broader cell line panels to more accurately model the full clinical landscape.

Second, the therapeutic agents investigated - cisplatin, vinblastine, and gemcitabine - are known to trigger diverse resistance mechanisms, many of which were beyond the scope of our targeted analysis. For instance, our investigation into cisplatin resistance focused on ATP7A, metabolic enzymes such as ASS1 and SAT1, and metallothionein-2 (MT2A)^[17,50-53]. However, we did not evaluate contributions from other critical pathways, including nucleotide excision repair (NER) via ERCC1, the roles of MLH1 and POLH in DNA damage tolerance, or the influence of autophagy^[13,33]. Similarly, while our analysis of vinblastine resistance highlighted the central role of the ABCB1 efflux pump and β 3-tubulin overexpression^[28,54], we only touched upon the distinct glutathione-based detoxification pathways that appear to differ between cell lines^[43]. Gemcitabine resistance further highlighted this limitation: although we analyzed key mediators such as *hENT1*, *dCK*, and *ECHDC1*, the most resistant cell line, UMUC-3^{Gem}, showed no significant changes in these genes, pointing toward an entirely alternative and uninvestigated resistance mechanism. One particularly intriguing possibility, not covered in our current study, is the involvement of cytidine deaminase (CDA), which converts the gemcitabine intermediate, 2',2'-difluorodeoxycytidine (dFdC), into its inactive metabolite, 2',2'-difluorodeoxyuridine (dFdU), along with other proteins associated with gemcitabine metabolism^[55,56].

Despite these limitations, our characterization provides useful landmarks for further research. By demonstrating that specific resistance mechanisms (e.g., p21/p27 modulation, ATP7A upregulation, or β 3-tubulin expression) are highly dependent on cellular context, our findings make it easier to select the most appropriate sensitive/resistant cell line pairs for hypothesis-driven studies. To build on this foundation and further delineate the underlying networks, future studies with these selected cell line pairs should employ unbiased, large-scale approaches. Comprehensive transcriptomic and proteomic analyses, in particular, would be invaluable for mapping the full range of molecular adaptations driving resistance, thereby uncovering novel therapeutic targets and strategies to overcome them.

In summary, our findings strongly suggest that a cell's foundational oncogenic drivers dictate its subsequent evolutionary path toward chemoresistance. This provides a molecular rationale for the heterogeneity of clinical responses and underscores the importance of genomic profiling, not only for selecting initial targeted therapies but also for anticipating and counteracting acquired resistance mechanisms.

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Availability of data and materials

Cell lines from this study are available for sharing upon reasonable request to Michaelis M (M.Michaelis@kent.ac.uk) or Cinatl J Jr. (j.cinatl@kinderkrebsstiftung-frankfurt.de). Data are contained within the article.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

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Not applicable.

Consent for publication

Not applicable.

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Resistance signatures manifested in early drug response across cancer types and species

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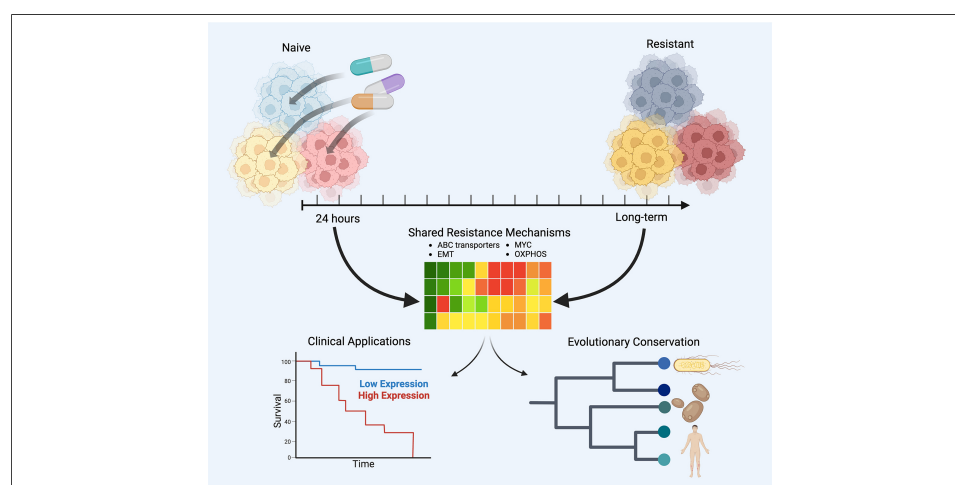
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Abstract

Aim: Growing evidence points to non-genetic mechanisms underlying long-term resistance to cancer therapies. These mechanisms involve pre-existing or therapy-induced transcriptional cell states that confer resistance. However, the relationship between early transcriptional responses to treatment and the eventual emergence of resistant states remains poorly understood. Furthermore, it is unclear whether such early resistance-associated transcriptional responses are evolutionarily conserved. In this study, we examine the similarity between early transcriptional responses and long-term resistant states, assess their clinical relevance, and explore their evolutionary conservation across species.

Methods: We integrated datasets on early drug responses and long-term resistance from multiple cancer cell lines, bacteria, and yeast to identify early transcriptional changes predictive of long-term resistance and assess their evolutionary conservation. Using genome-wide CRISPR-Cas9 knockout screens, we evaluated the impact of genes associated with resistant transcriptional states on drug sensitivity. Clinical datasets were analyzed to explore the prognostic value of the identified resistance-associated gene signatures.

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Results: We found that transcriptional states observed in drug-naïve cells and shortly after treatment overlapped with those seen in fully resistant populations. Some of these shared features appear to be evolutionarily conserved. Knockout of genes marking resistant states sensitized ovarian cancer cells to Prexasertib. Moreover, early resistance gene signatures effectively distinguished therapy responders from non-responders in multiple clinical cancer trials and differentiated premalignant breast lesions that progressed to malignancy from those that remained benign.

Conclusion: Early cellular transcriptional responses to therapy exhibit key similarities to fully resistant states across different drugs, cancer types, and species. Gene signatures defining these early resistance states have prognostic value in clinical settings.

INTRODUCTION

Although numerous chemotherapeutic, targeted, and monoclonal antibody-based drugs have been approved for various cancers, resistance invariably emerges, resulting in relapse and, ultimately, patient death. In fact, therapeutic resistance remains the leading cause of treatment failure and cancer-related mortality^[1]. Several mechanisms underlying resistance have been identified^[2]. While mutation-driven resistance has traditionally been the predominant paradigm^[3], recent evidence highlights an epigenetic or transcriptional basis for resistance. In this model, resistance arises from a subpopulation of malignant cells that adopt drug-resistant transcriptional states^[4,5]. These pre-existing, reversible resistant cells - referred to as drug-tolerant persister (DTP) cells^[6] - are distinct from stable, emergent resistant states^[7].

Recent studies have begun to characterize these resistant transcriptional states^[8,9], but several key questions remain. For instance, it is unclear whether, and to what extent, long-term transcriptional resistance is an inherent cellular response that becomes evident shortly after drug exposure. Additionally, the extent to which early cellular responses are shared across cancer types and drugs - and whether these responses are evolutionarily conserved - remains unknown^[10]. Identifying such broadly conserved early responses may hold clinical utility. These key questions form the basis of our investigation.

To address these questions, we first derived a broad, long-term resistance signature from previously published studies involving multiple cell lines and drugs^[9]. We then analyzed single-cell transcriptomic data capturing the early transcriptional response to drug treatment^[11]. By comparing these early response scRNA-seq data with the long-term resistance-associated transcriptional signature, we identified features of long-term resistance that are already apparent in early response states. Furthermore, we assessed the evolutionary conservation of these early resistance-associated responses using previously published transcriptional datasets from the bacterium *Escherichia coli* (*E. coli*)^[12] and the fungus *Candida auris* (*C. auris*)^[13].

Our findings indicate that transcriptional states associated with long-term resistance are already evident shortly after drug exposure. Across all three cell lines analyzed, we observed early transcriptional responses that closely resembled long-term resistant states. Notably, our analyses also suggest that these resistance-associated states may pre-exist prior to treatment, in addition to being induced by drug exposure. These early and pre-existing states share common features across cell lines, including elevated oxidative phosphorylation, epithelial-mesenchymal transition (EMT), hypoxia signaling, and MYC pathway activity. Interestingly, similar transcriptional states were observed in *E. coli* and *C. auris* following exposure to various drugs, suggesting that this mode of resistance may represent an evolutionarily conserved mechanism. Using a genome-wide CRISPR screen, we demonstrated that knocking out markers of the early resistance signature

increases drug sensitivity. Finally, we show that these resistance-associated early transcriptional signatures have prognostic relevance across multiple cancer types and clinical contexts. Specifically, they can distinguish responders from non-responders and differentiate premalignant breast lesions that progress to malignancy from those that remain benign.

METHODS

Creating the resistance signature

We developed a 72-gene drug resistance gene signature by aggregating ranked gene lists from previously conducted drug treatment experiments in six cell lines (COLO829, A375, HT29, MMACSF, EFM192A, and BT474)^[9]. These cell lines were exposed to drugs for 10 days, after which the surviving cells were sequenced. For each cell line, we identified differentially expressed genes between day 10 and day 0 using the FindAllMarkers() function from Seurat (v5.2.1)^[14]. Genes that were significantly upregulated ($\log_2FC > 0$, adjusted $P < 0.05$) were selected and ranked by $\log_2FC$. We then used Robust Rank Aggregation (RRA; v1.2.1)^[15] to generate a single ranked list and retained 72 genes with an adjusted rank P -value < 0.05 .

To validate this signature, we used time-course data from drug resistance development in PC9 lung cancer cells treated with Osimertinib^[9], with single-cell transcriptomics conducted at days 0, 3, 7, and 14. At each time point, fold changes for each gene were computed relative to all other time points using Seurat's FoldChange() function. Enrichment of the 72-gene resistance signature was then evaluated at each time point using Gene Set Enrichment Analysis (GSEA) via the fgsea R package (v1.32.4)^[16].

Single-cell dataset processing

For the Oren *et al.* dataset^[9], raw count matrices were downloaded from the public repository cited in the original publication (GEO: GSE150949). Cells with fewer than 200 or more than 2,500 detected features were excluded. Data were normalized via log transformation using a scale factor of 10,000.

For the Srivatsan *et al.* dataset^[11], filtered count matrices were downloaded from GEO (GSE139944). Genes expressed in fewer than 100 cells were removed to retain biologically relevant genes. Normalization was performed as above. Pre- and post-treatment cells were processed identically to minimize batch effects.

Functional enrichment analysis

Pathway enrichment analysis was conducted using the clusterProfiler R package (v4.14.6)^[17], including pathways from MSigDB^[18] hallmarks, intratumoral heterogeneity meta-programs (ITH MPs)^[19], and Gene Ontology (GO) biological processes. The enricher() function was used for hallmarks and MPs, while enrichGO() was used for GO terms. The number of overlapping genes between each pathway and the gene set of interest was reported as counts.

AUCell threshold

For all applications of AUCell (v1.28)^[20], we estimated an activation threshold for a given gene signature by generating a background distribution of AUCell scores using 100 control gene sets with expression levels matched to the target genes. These control gene sets were constructed following a procedure similar to Seurat's AddModuleScore() function.

Odds ratio for resistance signature activity in cell clusters

We computed the odds ratio for resistance signature activity in each cell cluster using Fisher's exact test. Clusters with an odds ratio > 1 and an adjusted P -value < 0.05 were defined as resistance-activated clusters (RACs). Fold enrichment for a drug class was calculated relative to the random expectation.

Global RAC gene signatures

To define global RAC signatures, we used Seurat's FindAllMarkers() function to compare all RAC cells to non-RAC cells within each cell line. Genes with an adjusted P -value < 0.05 and $\log_2FC > 0$ were retained. The top 200 genes, ranked by $\log_2FC$, were selected as the signature.

Grouping the RACs across cell lines into superclusters

We compiled a list of 3,084 genes, representing the intersection of the top 2,000 most variable genes in each cell line. For each gene, we calculated the differential mean expression in RACs relative to other cells within each line. RACs were then hierarchically clustered based on pairwise Spearman correlation of these gene expression vectors. Superclusters were defined as groups of highly correlated RACs containing components from at least two of the three cell lines.

Supercluster consensus signature creation

To generate a consensus signature for each supercluster, we followed the same approach as used for the initial resistance signature. For each RAC within a supercluster, we selected differentially expressed genes ($\log_2FC > 0$, adjusted $P < 0.05$), ranked them by $\log_2FC$, and aggregated the lists using RRA, retaining genes with an adjusted rank P -value < 0.05 . The same method was applied to identify downregulated genes ($\log_2FC < 0$).

Supercluster cell cycle phase plots

Cell cycle phase assignment within each supercluster was performed using Seurat's CellCycleScoring() function, incorporating an additional G0 phase gene signature^[21]. Each cell was assigned to the phase corresponding to its highest-scoring signature.

Drug class-specific superclusters

To determine whether cells treated with a specific drug class were overrepresented in a supercluster, we calculated the odds ratio comparing the proportion of treated cells within the supercluster's component clusters to the proportion outside those clusters.

Origin and maintenance of OVCAR8 cells

The OVCAR8 cell line, derived from the ascites of a patient with progressive high-grade serous ovarian carcinoma, was obtained from the NCI Developmental Therapeutics Program (DTP). Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and maintained at 37 °C in a humidified incubator with 5% CO₂. Mycoplasma contamination testing was performed regularly.

CRISPR screening

A genome-wide Brunello CRISPR knockout (KO) lentiviral library (Addgene #73178, Watertown, MA, USA) was used to identify genes conferring resistance to the CHEK1/2 inhibitor Prexasertib. The library includes 76,441 gRNAs targeting 19,114 genes and 1,000 non-targeting controls. OVCAR8 cells stably expressing Cas9 (Addgene # 52962-LV) were transduced at an MOI of ~0.3, followed by puromycin selection (5 µg/mL for three days). The selected population was divided into treatment (30 nM Prexasertib) and control (DMSO) arms, each cultured for 10 generations with 500× coverage. Genomic DNA was extracted using the QIAmp DNA Blood Cell Maxi Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. The sgRNA barcodes were PCR-amplified using Taq polymerase and adapted for sequencing. The desired DNA product was purified using a 6% TBE gel (Invitrogen, Waltham, MA, USA) and the samples were sequenced on an Illumina HiSeq2000. Read counts and hit identification were performed using MAGeCK (v0.5.7)^[22], with gene ranking based on RRA and normalization to non-targeting gRNA distributions.

Kaplan-Meier plots

Kaplan-Meier survival analysis was conducted using the *survminer* R package (v0.5.0)^[23]. Samples were divided into high and low expression groups using the top and bottom 50th percentiles of ssGSEA scores, derived from the GSVA R package (v2.0.7)^[24]. Survival time is shown in days, and *P*-values were calculated using the log-rank test.

Cox regression

Cox proportional hazards regression was performed using the *survival* R package (v3.8-3)^[25]. Overall survival was modeled using ssGSEA scores for the resistance signature, age, sex, and tumor purity (when available).

Scoring recurrence, pre-malignancy, and drug response data

For recurrence, pre-malignancy, and drug response analyses, patient samples were scored using ssGSEA with the global RAC or supercluster signatures.

Creating resistance ortholog signatures

To construct a drug resistance signature for *C. auris*, we retrieved differential gene expression data comparing resistant and sensitive populations from the original study^[13], selecting genes with $\log_2\text{FC} > 0$ and adjusted $P < 0.05$. For *E. coli*, we used expression data from nine experiments involving various antimicrobials^[12], identifying upregulated genes ($\log_2\text{FC} > 0$, adjusted $P < 0.05$) in each. Genes present in at least 5 of the 9 experiments were selected. Orthologs were mapped to the human genome using OMA Browser^[26].

Scoring for resistance ortholog signatures in superclusters

Cells from early response data were scored for the *C. auris* and *E. coli* ortholog resistance signatures using AUCell. Score distributions were compared using the Wilcoxon rank-sum test.

Functional enrichment of conserved genes

To assess the biological relevance of conserved resistance genes, we identified overlaps between the *C. auris* or *E. coli* resistance signatures and supercluster signatures with enriched AUCell scores. These overlapping genes were analyzed for enrichment in MSigDB hallmarks, ITH MPs, and GO processes as described above.

RESULTS

Multiple cell states in pre-treatment and early response data express long-term resistance programs

To identify early post-treatment manifestations of long-term drug resistance, we developed a gene signature composed of commonly upregulated genes [Supplementary Table 1] shared across six drug-resistant samples from previous studies^[9]. These resistance studies involved a wide variety of cancer cell lines treated with different drugs, and thus, the derived consensus gene set represents a general drug resistance program. We validated this gene signature using an independent dataset that included multiple timepoints during the development of resistance in a different cancer type - lung adenocarcinoma^[9]. Notably, we observed strong enrichment of the signature at day 14 [Figure 1A], supporting the generality of the derived resistance program. Functional enrichment analysis [Supplementary Figure 1A] revealed the involvement of known resistance-associated pathways such as EMT and hypoxia^[27,28].

Next, we analyzed an independent, large-scale dataset containing 581,308 cells, including both drug-naïve and 24-hour post-treatment cells from three cancer cell lines (A549, K562, MCF7) treated with 188 compounds across 15 drug classes^[11]. Notably, these three cell lines differ from the six used to derive the long-term resistance signature. For each of the three cell lines, we independently assessed the relative expression of the consensus resistance signature within each transcriptional cluster. In every cell line, certain

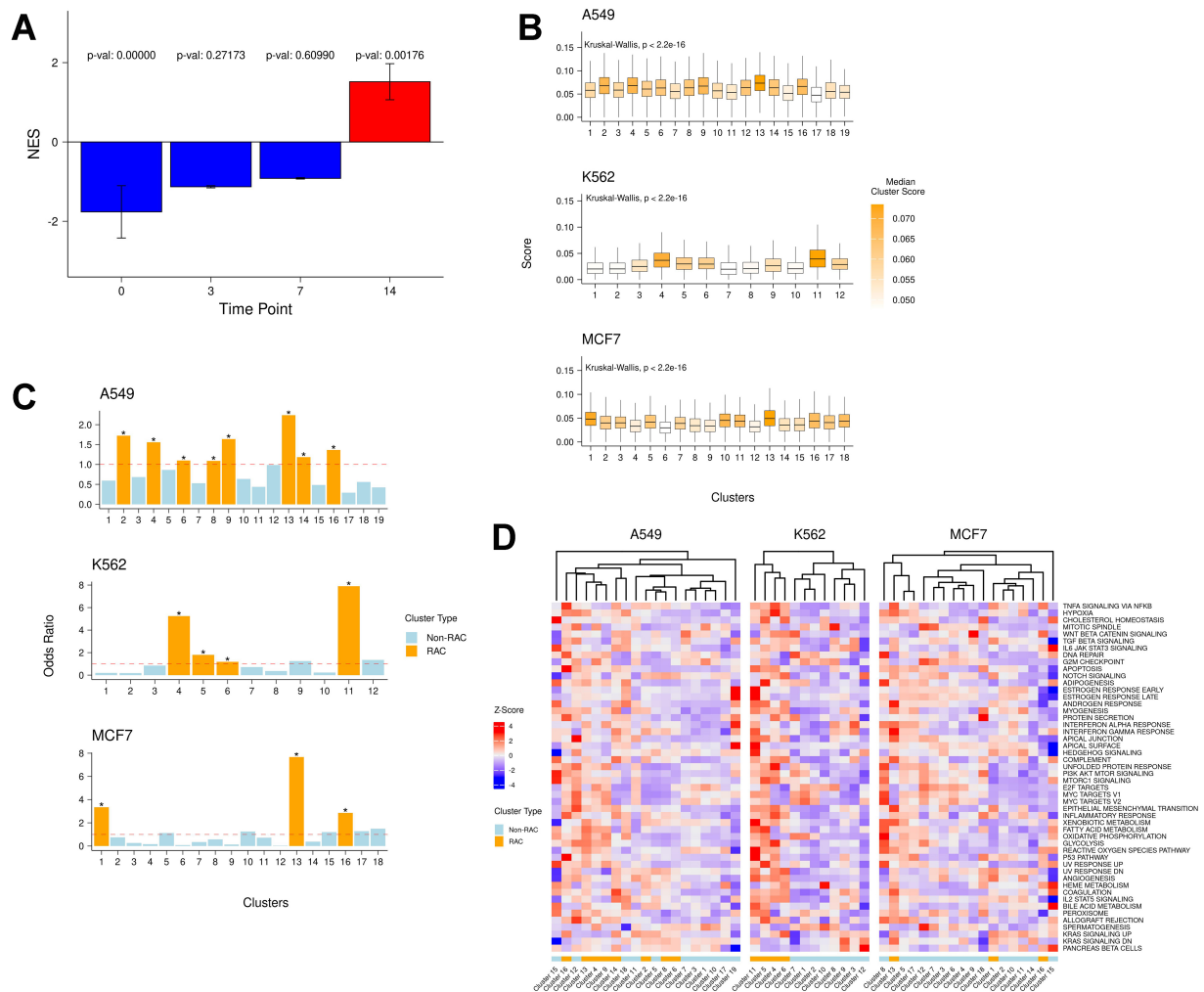


Figure 1. (A) Enrichment of the derived drug resistance signature gene at days 0, 3, 7, and 14 in the Oren et al. dataset^[9]; (B) Distributions of raw AUCell scores of the resistance signature in each cluster across all cell lines; (C) Enrichment odds ratio of resistance-active cells in each cluster. Asterisks indicate significant odds ratio values ($P < 0.05$); (D) Standardized (z-score) mean AUCell scores of cancer hallmark pathways in each cluster across all cell lines. NES: Normalized Enrichment Score.

clusters exhibited significantly higher resistance signature scores [Figure 1B], suggesting either the pre-existence of resistance-associated gene expression or its induction within 24 h of drug treatment.

To identify the cell states that may represent early manifestations of resistance, we evaluated the relative enrichment of resistance-active cells in each cluster compared to all other clusters within the same cell line [Figure 1C]. Multiple clusters in each cell line were significantly enriched for resistance-active cells, which we designated as RAC - 15 in total.

Moreover, in each cell line, some clusters exhibited high levels of resistance signature expression even before treatment, consistent with pre-existing resistance^[7] [Supplementary Figure 1B]. Collectively, these findings indicate that multiple cell states, both pre-treatment and within the early drug response phase, express resistance programs resembling those observed in long-term drug-resistant samples.

RACs are enriched for conserved resistance pathways across cell lines

Having identified RACs in each cell line, we next aimed to functionally characterize them using cancer hallmark signatures (from MsigDB^[18]) and intratumor heterogeneity meta-programs (ITH MPs)^[19] [Figure 1D and Supplementary Figure 1C]. In two of the three cell lines, most RACs clustered together

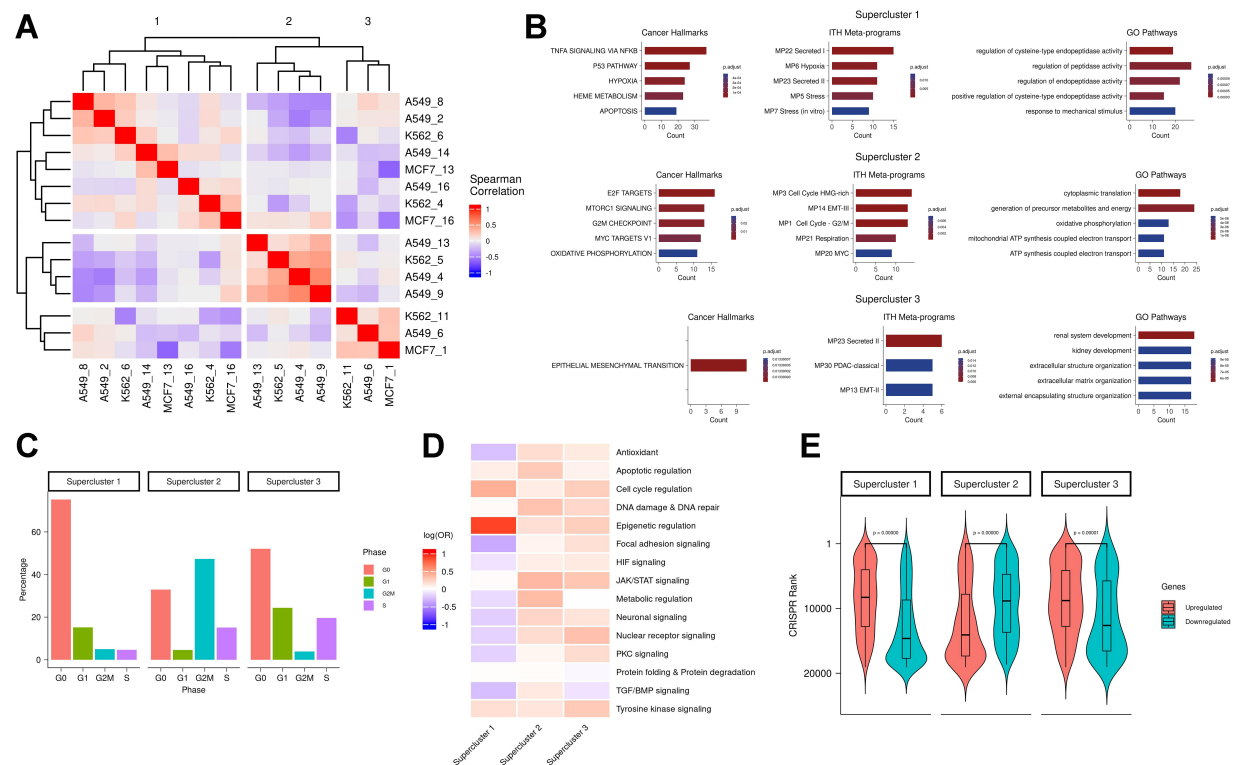


Figure 2. (A) All-by-all Spearman correlation matrix of RAC differential mean expression vectors; (B) Functional enrichment of supercluster signatures. Columns from left to right show enrichment in cancer hallmarks, ITH MPs, and GO pathways, respectively. Rows represent the three superclusters. Count values indicate the number of overlapping genes between the supercluster signature and the enriched pathway; (C) Distribution of cell cycle phase percentages for each supercluster (G0 = red; G1 = green; G2M = blue; S = purple); (D) Drug class enrichment for each supercluster; (E) CRISPR rank distributions (Y-axis) for upregulated (red) and downregulated (blue) genes in each supercluster. RAC: Resistance-activated cluster; ITH MPs: intratumoral heterogeneity meta-programs; GO: Gene Ontology.

hierarchially, although there was some diversity in hallmark activity among RACs within each line. This is consistent with previously reported distinct and functionally divergent resistant states^[8]. Notably, in each cell line, RACs exhibited both high and low enrichment of cell cycle-related signatures, aligning with earlier observations of variability in cell cycling among resistance states^[9]. A similar pattern emerged when we analyzed gene enrichment between all RAC and non-RAC cells in each cell line, regardless of clusters (Supplementary Materials - “Global RAC Signature Enrichment”).

To define pan-cell line resistance signatures, we grouped all 15 RACs from the three cell lines into three superclusters based on their transcriptional similarities [Figure 2A and Supplementary Figure 2A]. Each supercluster included RACs from at least two cell lines. To further probe their function, we generated two gene sets per supercluster by aggregating genes consistently upregulated or downregulated in all component RACs from each line [Supplementary Table 1].

Functional enrichment analysis of the upregulated genes in each supercluster revealed both shared and distinct enriched programs across the three superclusters [Figure 2B]. Supercluster 1 was enriched for hypoxia and TNF α signalling - pathways previously implicated in therapy resistance across various cancer types^[29]. Hypoxia contributes directly to drug resistance by reducing drug cytotoxicity^[28] and promoting cellular plasticity, which enables adaptive survival responses^[30]. Supercluster 2 was enriched for MYC activity, a key driver of drug resistance^[31,32], and showed metabolic rewiring^[33] characterized by enrichment in respiration and oxidative phosphorylation pathways. Supercluster 3 was enriched for EMT and secretion pathways, both commonly associated with resistance mechanisms^[27,34]. Enrichment analysis of the

downregulated gene signatures for each supercluster is provided in the [Supplementary Materials - “Supercluster downregulated gene signature enrichment”](#).

Pathways enriched in the supercluster 2 signature included several related to cell cycling progression, indicating a proliferative resistant state. This was supported by notable differences in the distribution of cell cycle phases among superclusters [Figure 2C], suggesting that resistant states differ in their proliferation rates - likely due to metabolic variation^[9]. In particular, supercluster 1 exhibited the highest proportion of cells in the G0 phase, suggestive of a quiescent drug-resistant state^[35,36].

To investigate the influence of drug classes on resistance states, we analyzed the association between superclusters and specific drug treatments. Supercluster 1 consisted predominantly of cells treated with drugs targeting epigenetic regulation, while superclusters 2 and 3 included cells treated with a broader range of drug classes [Figure 2D]. These patterns suggest that certain drug classes may preferentially induce specific resistance states. Our findings support the hypothesis that some drugs trigger similar resistance states across different cancer types, implying that the transcriptional response may be more dependent on the treatment than on the cancer type itself.

CRISPR knockout of resistance-associated genes sensitizes cells to drug treatment

Here, we experimentally validated the resistance gene signatures by testing whether genes defining the superclusters have a predictable impact on drug resistance in a novel context. We selected a cancer type and drug that were not included in any of the previously analyzed datasets, and assessed whether knocking out genes characteristic of each supercluster would increase sensitivity to cytotoxic drugs. To this end, we performed a genome-wide CRISPR knockout screen in the ovarian cancer-derived cell line OVCAR8 and measured the effect of individual gene knockouts on cell viability following treatment with the CHK1/2 checkpoint kinase inhibitor Prexasertib over 10 cell generations. Genes were ranked based on the degree of increased drug sensitivity upon knockout; higher-ranking genes in this list are more likely to mediate drug resistance. Encouragingly, we found that in superclusters 1 and 3, the upregulated genes exhibited significantly higher CRISPR-based ranks than the downregulated genes (P -value ≈ 0 for both; Figure 2E). This finding - that upregulated genes in the low-proliferation states (superclusters 1 and 3) scored higher in the Prexasertib screen - is consistent with the drug's known mechanism of action, namely replication catastrophe^[37]. Conversely, in supercluster 2, which is characterized by a proliferative state, knockout of the upregulated genes led to reduced sensitivity to the drug [Figure 2E]. These results suggest that disrupting transcriptional states associated with resistance in the early response phase could offer a viable strategy for modulating drug tolerance.

Conserved resistance states have clinical relevance

Here, we aimed to evaluate the clinical relevance of cell line-specific global RAC signatures and the RAC supercluster gene signatures. First, for each cell line, we assessed the association between the cell line's global RAC signature and patient survival in the corresponding cancer type using data from TCGA. We scored the patient samples with the respective signature and performed Cox regression analysis on overall survival. As shown in Figure 3A, the Kaplan-Meier plot for the K562 RAC signature applied to the LAML cohort suggests that high expression of the global RAC signature is associated with poorer survival. Although the results are not statistically significant, all three cases demonstrated a positive association between the RAC signature score and hazard ratio (A549-LUAD: HR = 2.1, P -value = 0.15; K562-LAML: HR = 2.5, P -value = 0.14; MCF7-BRCA: HR = 2.0, P -value = 0.27).

Because superclusters represent multiple cancer types, we repeated the above analysis using the supercluster signatures across all TCGA cohorts [Figure 3B]. Despite considerable variability across cancer types, the distribution of log-transformed hazard ratios was significantly greater than zero (P -values = 0.0176, 0.0016, and 0.0082, respectively, for the three cell lines; one-sample t -test).

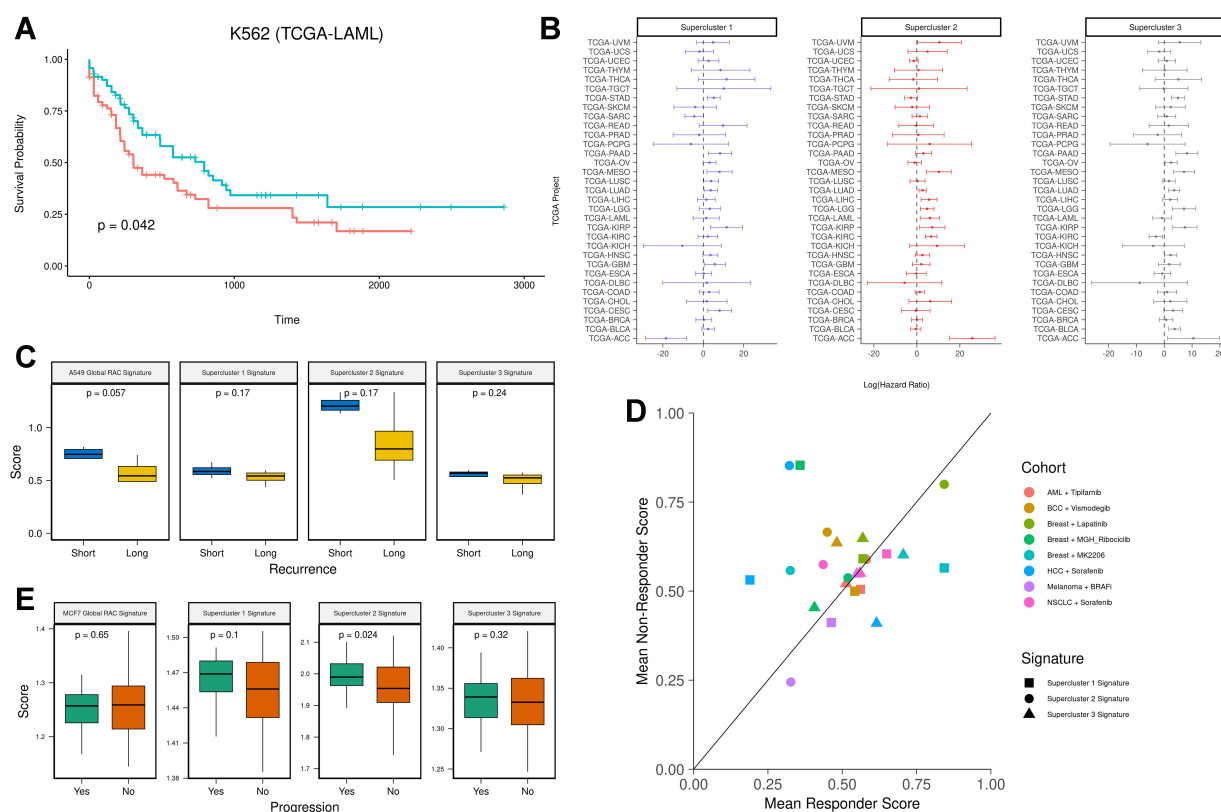


Figure 3. (A) Kaplan-Meier survival analysis of the K562 RAC signature in the LAML cohort. Patients were stratified into two groups based on K562 RAC signature scores (red = high, blue = low); (B) Hazard ratios with 95% confidence intervals for supercluster signatures across all 33 TCGA cancer types; (C) Pre-treatment RAC signature scores in lung cancer patients prior to tyrosine kinase inhibitor treatment. The Y-axis shows ssGSEA scores, and the X-axis stratifies patients by recurrence time (blue = short-term recurrence; yellow = long-term recurrence); (D) Mean supercluster signature scores for responders and non-responders across various treatment cohorts (cohort = drug × cancer type); (E) RAC signature scores in premalignant lesions. The Y-axis represents ssGSEA scores, and the X-axis stratifies patients by progression status (green = progression; orange = no progression). RAC: Resistance-activated cluster.

Next, to evaluate the predictive value of the supercluster signatures for therapy response, we analyzed a dataset comprising pre-treatment tumor samples from eight patients with EGFR-mutant lung cancer treated with the tyrosine kinase inhibitor Osimertinib^[38]. All patients had relapsed after initial response and were classified into two groups: short-term recurrence (< 8.6 months; $n = 4$) and long-term recurrence (≥ 12 months; $n = 4$). Each pre-treatment sample was scored using the A549 global RAC signature and the three supercluster signatures. We found that patients with short-term relapse exhibited higher resistance scores across all signatures ($n = 8$, Figure 3C). Statistical significance was achieved in one case, likely due to the small sample size. These findings suggest the presence of pre-existing resistance mechanisms in short-term responders.

We then assessed whether the supercluster signatures could predict therapy response across multiple cancer types and drug regimens. We analyzed pre-treatment bulk RNA sequencing and microarray datasets spanning six cancer types and seven drug treatments, in which patients were categorized as responders or non-responders^[39] [Supplementary Table 3]. On average, non-responders exhibited higher supercluster signature scores across all cohorts, although the difference did not reach statistical significance (one-sided t -test, P -value = 0.08; Figure 3D).

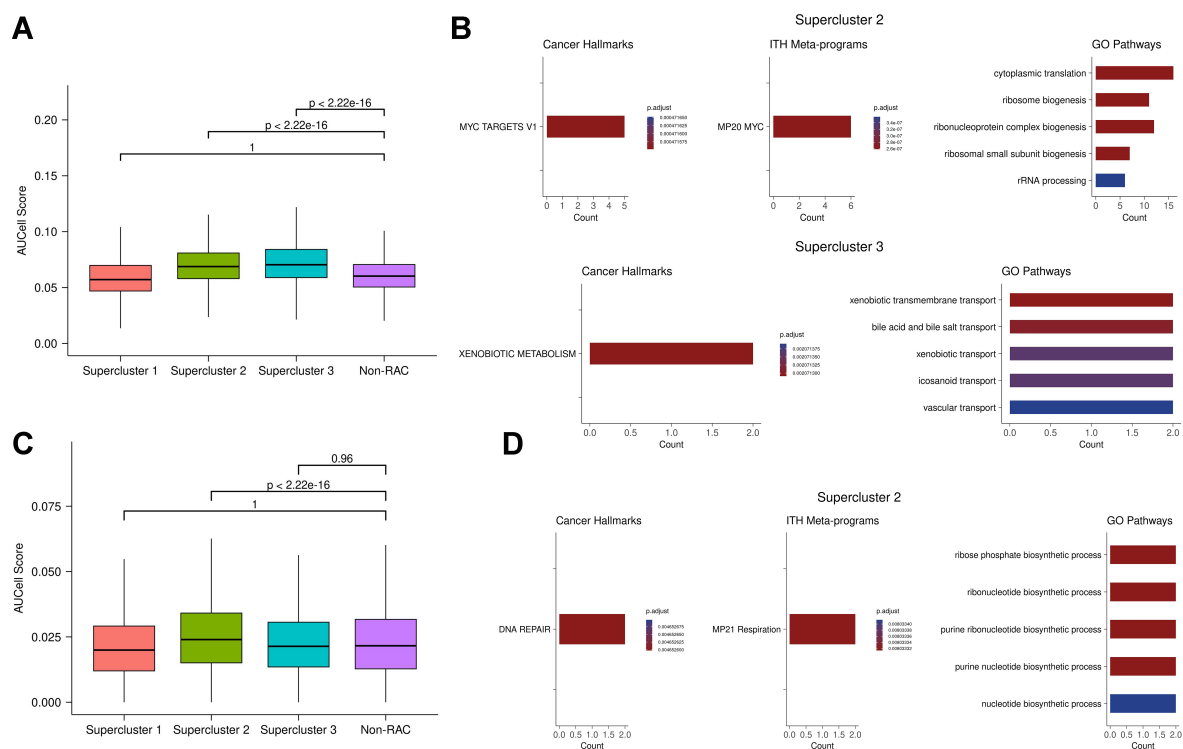


Figure 4. (A) *C. auris* antifungal resistance gene signature scores in human resistance and non-resistance clusters. Each distribution shows scores for individual cells in each supercluster or non-RAC cluster across three cell lines; (B) Pathway enrichment analysis of shared genes between *C. auris* resistance orthologs and superclusters 2 and 3. “Count” indicates the number of overlapping genes between the gene set of interest and the enriched pathway; (C) *E. coli* antimicrobial resistance gene signature scores in human resistance and non-resistance clusters. Each distribution shows scores for individual cells in each supercluster or non-RAC cluster across three cell lines; (D) Pathway enrichment analysis of shared genes between *E. coli* resistance orthologs and supercluster 3. RAC: Resistance-activated cluster.

Finally, we examined whether supercluster signatures are associated with the progression of premalignant lesions. To this end, we analyzed a dataset from the HTAN database^[40,41] that includes 97 premalignant breast lesion samples, of which 31 progressed to cancer and 66 did not. We scored each sample using the MCF7 global RAC signature and the three supercluster signatures. Samples from the progression group showed higher scores, with statistical significance observed only for the supercluster 2 signature [Figure 3E]. Consistently, we also found that genes differentially expressed in the progression group were significantly enriched in the supercluster 2 signature [Supplementary Figure 2C]. These results suggest that resistance-associated signatures are linked, to some degree, with malignant transformation.

Supercluster resistance mechanisms are evolutionarily conserved

Having identified early cellular responses broadly associated with resistance, we hypothesized that these resistance-related transcriptional responses reflect an inherent cellular reaction to chemically induced stress and are therefore evolutionarily conserved across species. To test this, we retrieved a gene signature comprising genes upregulated in the antifungal-resistant yeast *C. auris*^[13] and mapped them to their human orthologs^[26] [Supplementary Table 4]. We then scored all cells in the early-response dataset and compared score distributions between supercluster cells and non-RAC cells. In superclusters 2 and 3, cells exhibited significantly higher scores for the *C. auris* resistance ortholog signature compared to non-RAC cells, with Cohen’s d effect sizes of 0.58 and 0.70, respectively. This suggests that superclusters 2 and 3 may represent conserved drug response mechanisms shared between yeast and humans [Figure 4A].

To further investigate the underlying shared mechanisms, we performed functional enrichment analysis on genes common to the *C. auris* resistance ortholog set and the genes upregulated in superclusters 2 and 3

[Figure 4B]. This analysis revealed enrichment for MYC activity and ribosome biogenesis, suggesting that these processes are evolutionarily conserved responses to chemical stress and may contribute to resistance. The observed increase in MYC activity and ribosome biogenesis aligns with previous studies showing that MYC is a key regulator of ribosome biogenesis^[42,43], and both processes are implicated in drug resistance in yeast and human cancers^[31,44,45]. Additionally, pathways enriched in the overlap between *C. auris* orthologs and supercluster 3 include xenobiotic metabolism and various transport mechanisms. Notably, genes such as ABCC2 and ABCC3 (orthologous to the *C. auris* gene B9J08_002146) contribute to this enrichment. These genes encode ATP-binding cassette (ABC) transporters, which are known to drive multidrug resistance (MDR) in both yeast and human cancer^[46,47]. Taken together, these findings suggest that resistance mechanisms conserved between *C. auris* and human cancer involve MYC, its downstream regulatory processes, and drug efflux pumps.

To explore conservation of resistance mechanisms across additional species, we analyzed expression data from drug-treated *E. coli* strains. We identified genes upregulated in resistant versus drug-naïve conditions across nine antimicrobial treatments and compiled a consensus antimicrobial resistance gene set [Supplementary Table 4]. These *E. coli* resistance genes were then mapped to their human orthologs, and we scored all human cells for the resulting gene signature. In supercluster 2, resistance scores were significantly higher than in non-RAC cells, with an effect size of 0.16 [Figure 4C].

We then performed pathway enrichment on genes shared between the *E. coli* resistance ortholog set and those upregulated in supercluster 2 [Figure 4D]. The enriched pathways suggest that DNA repair is a conserved resistance mechanism between *E. coli* and human cancer. This is consistent with previous studies demonstrating that enhanced DNA repair contributes to resistance in both contexts^[48,49]. Overall, these analyses support the hypothesis that superclusters 2 and 3 represent evolutionarily conserved mechanisms of drug resistance. Specifically, superclusters 2 and 3 appear to reflect shared resistance pathways between humans and *C. auris*, involving the proto-oncogene MYC, its downstream targets such as ribosome biogenesis, and drug efflux systems. Additionally, supercluster 2 exhibits conserved resistance mechanisms between humans and *E. coli*, notably involving enhanced DNA repair.

DISCUSSION

In this study, we comprehensively assessed the extent to which transcriptional programs that characterize drug resistance in cancer cells are either pre-existing prior to treatment or emerge early in response to therapy. These early programs may represent intrinsic cellular responses to cytotoxic stress, some of which form the basis for long-term resistance. We found that transcriptional programs typically observed in established resistance - such as EMT^[27,50], MYC signaling^[31,32], and oxidative phosphorylation^[33] - are already detectable just 24 h after drug treatment. Moreover, these early manifestations are not limited to a single cancer type; they are evident across multiple cancer cell lines. Notably, aspects of these responses show potential evolutionary conservation in bacteria and yeast, suggesting that such intrinsic resistance programs may represent a fundamental cellular survival mechanism conserved across species. Previous studies have shown that genes with high expression variability often emerge as differentially expressed across unrelated experiments and are enriched for stress-response functions^[51,52]. Ultimately, causality must be established through targeted experimental approaches. Using a whole-genome CRISPR knockout screen, we validated that disrupting genes associated with early resistance states increases drug sensitivity in a manner consistent with the known mechanisms of action of the respective drugs. Further validation, including longitudinal lineage tracing under perturbation, could strengthen these findings and confirm causal roles for the identified gene signatures. Importantly, these early response signatures also show prognostic value in premalignant states and overall survival, suggesting that they may reflect cell states inherently resistant to diverse stressors that cells have faced throughout evolution.

It is well established that many cancer treatments compromise immune infection, increasing infection risk in patients with advanced disease^[53,54]. Our finding that components of the early drug response are conserved across species raises the possibility of developing therapies that simultaneously target cancer resistance and pathogen resistance. By focusing on conserved resistance mechanisms, such therapies could improve treatment efficacy while lowering infection-related mortality^[54].

Consistent with previous reports^[8,9], our analyses reveal distinct modes of resistance. These resistance-associated transcriptional states, conserved across cancer types, differ in their cell cycle status and are associated with specific drug classes [Figure 2]. Remarkably, even in pre-treatment tumor transcriptomes, these early response signatures correlate with relapse timing, overall survival, therapy response, and tumor progression across a range of cancers and therapies [Figure 3]. Since the data used for these predictions were obtained at diagnosis, before chemotherapy, it is likely that the resistant states were already present and subsequently selected for during treatment. Conversely, other transcriptional states appear only after treatment, suggesting they may represent adaptive responses [Figure 1C and Supplementary Figure 1B]. Our findings corroborate recent reports on DTP cells, which are rare, slow-cycling cells that transiently persist post-treatment and can resume proliferation upon drug withdrawal, becoming re-sensitized to the original therapy^[6,55]. However, whether DTPs are pre-existing or arise via therapy-induced reprogramming remains unclear^[7], and this question could be best addressed through lineage tracing. Additionally, rare pre-existing resistant populations might evade detection in single-cell profiling due to limited sensitivity for capturing low-frequency states, leading them to appear as emergent after treatment.

Although we observed consistent expression of resistance signatures across several cancer types, their robustness could be improved. Our initial resistance signature was derived from only six datasets, and the early response signature from just three cell lines. Expanding this analysis to include additional cancer types could enhance the generalizability of these signatures and potentially uncover resistance mechanisms not captured here. Tumor-specific variations in the tumor microenvironment - particularly *in vivo* or in organoid models - likely contribute to additional resistance programs absent in our current analysis. Moreover, many of our conclusions are based on correlational signature scoring; hence, future CRISPR-based functional studies will be needed to validate the biological causality of these associations.

Our drug response dataset includes a wide range of drugs and classes, lending broad relevance to the identified resistance-associated signatures^[11]. One well-known mechanism underlying MDR involves ABC transporters. We found that all three major gene signatures - cell type-specific global RAC, supercluster 1, and supercluster 3 - include ABC transporter genes (e.g., ABCA1, ABCA12, ABCB1, ABCC2, ABCC3, ABCC13, and ABCG1), indicating their potential role in resistance across cell lines. While P-glycoprotein 1 (Pgp, ABCB1) has been shown to be inducible by drug treatment^[56], the extent of its involvement in the resistance observed in our study remains to be determined.

In conclusion, we identified multiple transcriptional states associated with non-specific drug resistance mechanisms across various cancer types and even across species such as *E. coli* and *C. auris*. The gene signatures derived from these resistant states offer promising avenues for further investigation^[57]. Although more research is needed to fully elucidate the mechanisms of resistance and strategies for their inhibition, our findings contribute to a growing understanding of the molecular features of early resistant transcriptional states. These insights may ultimately inform the development of novel adjuvant therapies to prevent or overcome resistance.

DECLARATIONS

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Authors' contributions

Conceptualization and design: Ruoff C, Hannenhalli S, Gopalan V

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Manuscript drafting: Ruoff C

Manuscript revision: Ruoff C, Hannenhalli S, Mitchell A, Mondal P, Gopalan V, Gottesman M, Singh A

Financial support: Hannenhalli S, Gottesman M

All authors contributed to and approved the article.

Availability of data and materials

All data needed to evaluate the conclusions in the paper are present in the paper and/or the [Supplementary Materials](#). Public datasets used in this study are available in the referenced papers. Source code and analysis scripts are available on GitHub: (https://github.com/coleruoff/drug_treatment).

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Conflicts of interest

Gottesman M is an Editorial Board member of the journal *Cancer Drug Resistance*. Gottesman M was not involved in any steps of editorial processing, notably including reviewer selection, manuscript handling, or decision making. The other authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

This study utilized de-identified data from the Gene Expression Omnibus (GEO) database, which is publicly accessible. The original collection and release of these data have already been approved by the respective institutional review boards of the contributing centers, in compliance with ethical guidelines and privacy regulations. As such, no additional ethics approval was required for the secondary analysis of these publicly available, de-identified data.

Consent for publication

Not applicable.

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Supplementary Materials

[Supplementary Materials](#)

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