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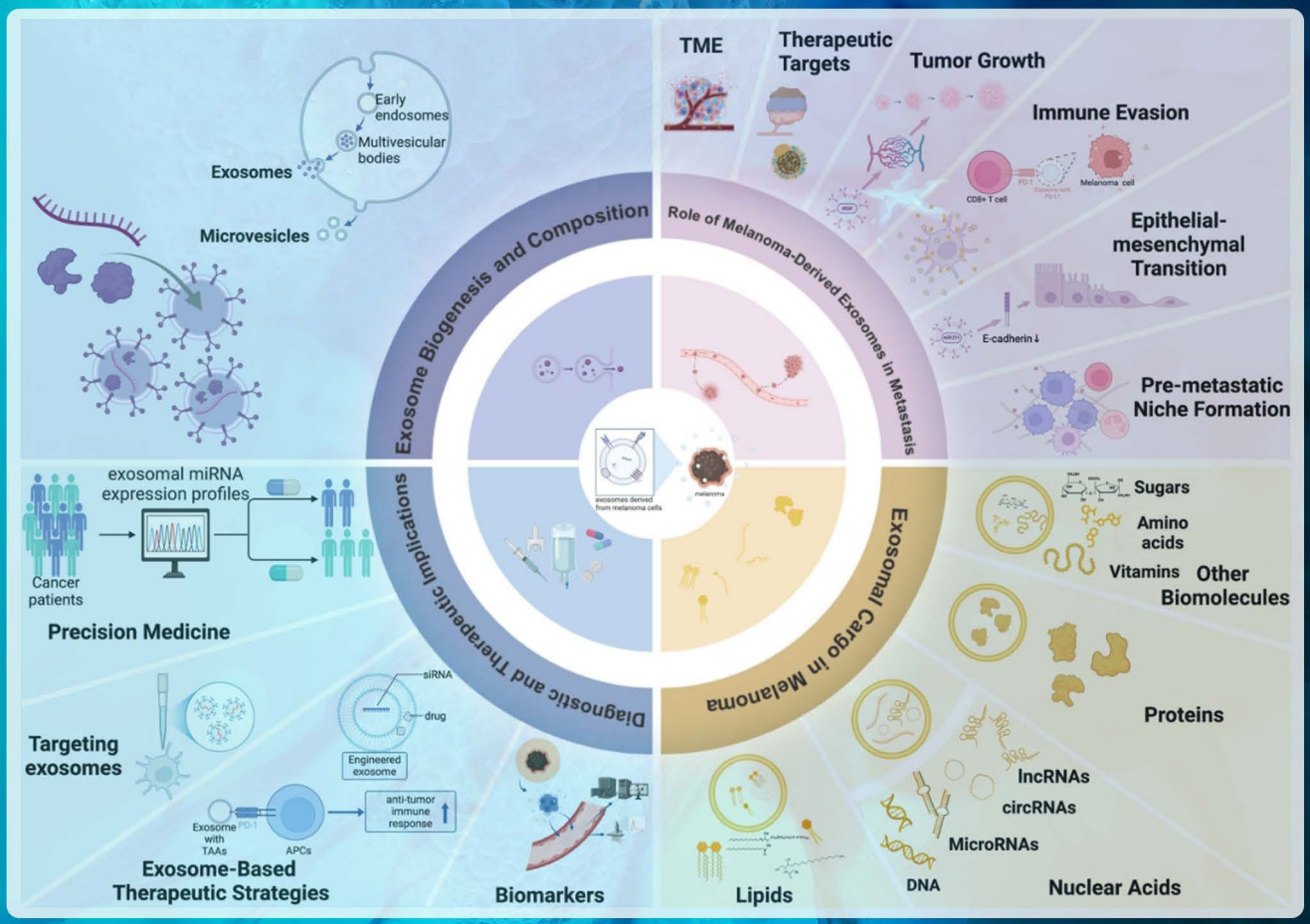

The role of melanoma-derived exosomes in metastasis: Challenges and opportunities

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EDITORIAL

The transformative role of AI in cancer research

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1. AI in the management of complex cancer data

Cancer research is inherently data-intensive. It integrates information from diverse domains such as genomics, proteomics, clinical records, and imaging, each providing unique insights into disease origins and progression. The volume and complexity of these data sets often exceed the capabilities of traditional analytical approaches. This is where artificial intelligence (AI) becomes a powerful ally.[1] AI excels at managing heterogeneous data sets. It processes raw data by performing essential tasks such as cleaning, normalization, and preprocessing. These steps are crucial, as cancer data sets often present issues such as missing data points and variations in format across studies and institutions. By automating these processes, AI reduces the risk of human error and speeds up the preparation of data for further analysis. Once preprocessed, the data must be analyzed to find patterns that can reveal biomarkers – indicators of disease presence or progression.[2] Machine learning algorithms, especially supervised and unsupervised learning models, are adept at this task. They can examine large amount of genomic and proteomic data to identify subtle patterns that correlate with specific types of cancer.[3] For example, machine learning models can link genetic mutations to certain cancer types, uncovering biomarkers that might not be apparent through traditional techniques. These biomarkers are invaluable in a variety of contexts, including early detection, prognosis prediction, and therapeutic targeting. Furthermore, AI-based predictive models can estimate a patient's response to specific treatments, allowing for personalized cancer therapy – a pillar of modern oncology.[4]

One of the most transformative applications of AI in cancer research lies in the analysis of genomic sequencing.[5] Genomic data holds clues about the mutations that drive cancer. Decoding this information is essential to understanding the disease and designing targeted interventions. Deep learning models have proven particularly effective in this arena. Deep learning excels at identifying subtle genetic mutations that traditional statistical methods might miss. These algorithms analyze sequence data to identify new genes associated with cancer, annotate genetic variants, and infer their functional significance. By unraveling these genetic mysteries, researchers can identify potential drug targets, laying the groundwork for innovative therapies. In addition, AI helps in building complex models of biological pathways. These models reveal intricate networks of genes and proteins, offering insights into the underlying mechanisms of cancer. Such pathways help researchers identify points of therapeutic intervention, paving the way for designing drugs that disrupt these networks and halt disease progression.

Medical imaging is another domain where AI is making significant progress.[6] Cancer diagnosis often relies on imaging technologies such as magnetic resonance imaging, computed tomography scans, and histopathological slides. Interpreting these images requires precision, as subtle features can indicate the presence or progression

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of the disease. AI-powered imaging tools, particularly convolutional neural networks, excel at detecting patterns in medical images. They can identify tumors, classify their types, and even predict their aggressiveness. These tools often outperform human radiologists in certain tasks, offering consistent and rapid analyses.

For example, in histopathology, AI algorithms analyze tissue samples to detect cancer cells.[7] They identify morphological patterns that may escape the human eye, increasing diagnostic accuracy. This capability is especially valuable in early detection, where timely intervention can significantly improve patient outcomes. The role of AI in drug discovery is another paradigm shift for cancer research.[8] Traditional drug development is notoriously slow and expensive, often taking more than a decade from discovery to approval. AI streamlines this process by identifying promising drug candidates more quickly. Through the analysis of biological pathways and protein interactions, AI algorithms highlight genes and proteins crucial to cancer development. By targeting these molecules, researchers can design therapies with a higher likelihood of success. In addition, AI facilitates drug repurposing, a process in which new uses for existing drugs are identified. This approach saves time and resources, as these drugs have already passed safety tests.

2. Future challenges and ethical considerations

Despite its transformative potential, the application of AI in cancer research is not without its challenges. A primary concern is data quality. Although AI can process large data sets, the conclusions it draws are only as reliable as the data it analyzes. Ensuring high-quality and representative data sets is critical to avoid biased or misleading results. Another challenge lies in interpretability. Many AI models, particularly deep learning systems, operate as "black boxes," producing results without offering clear explanations about how they were derived. This lack of transparency can hinder

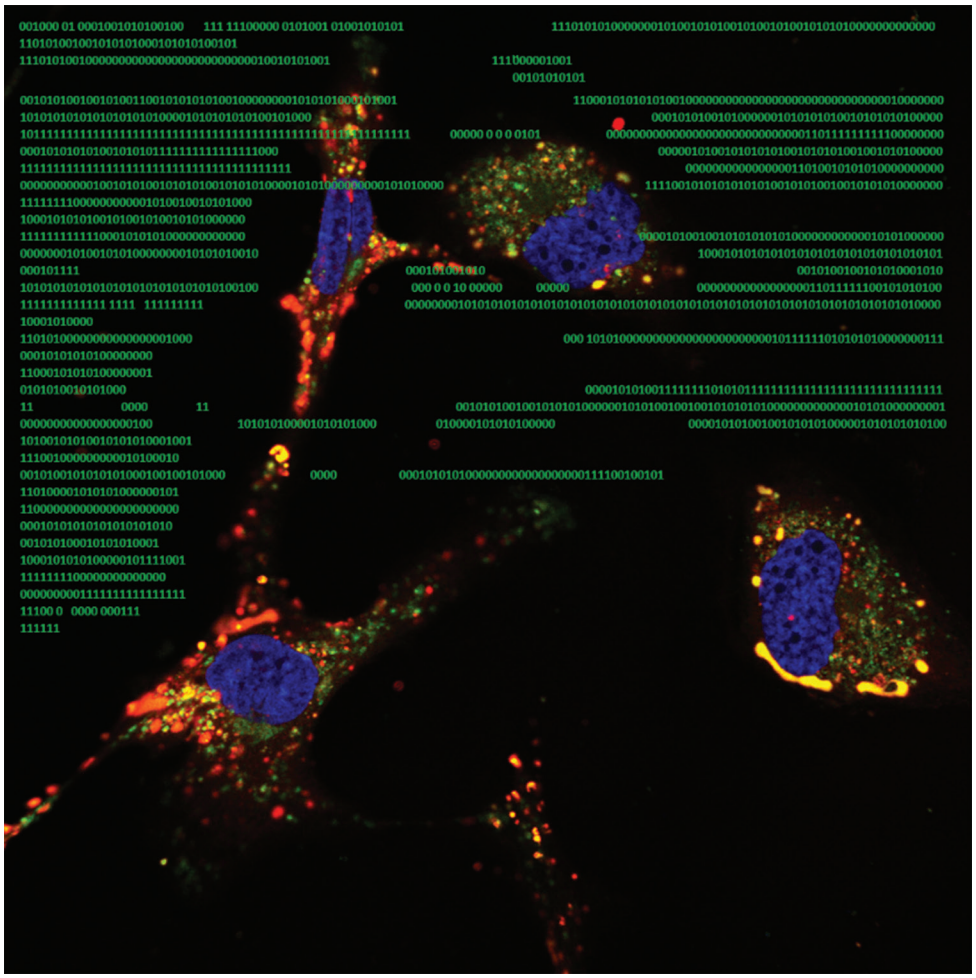

Figure 1. Imaginative representation of artificial intelligence in cancer research, showing the superimposition of a binary text in a background of a picture of tumor cells.

trust between researchers and clinicians, complicating the integration of AI into practice. Ethical considerations are also significant.[9] AI systems require access to large amounts of patient data, raising concerns about privacy and consent. Robust data governance frameworks are essential to protect patient confidentiality while enabling research. In addition, AI's potential to exacerbate health disparities must be addressed. If AI models are trained on data from specific populations, they may perform poorly in underrepresented groups, perpetuating inequities in cancer care. However, the future of AI in cancer research is undoubtedly promising. As computational power increases and algorithms become more sophisticated, AI capabilities will expand even further. One exciting front is multi-omic data integration, combining genomic, proteomic, transcriptomic, and metabolomic information to provide a comprehensive view of cancer biology.

Another area of growth is real-time monitoring and decision-making. AI-powered tools could continuously analyze patient data, offering physicians real-time insights into disease progression and treatment effectiveness. These capabilities would revolutionize personalized medicine, ensuring that interventions are tailored to each patient's unique needs. Collaborative efforts will also be crucial. Partnerships between AI researchers, oncologists, and pharmaceutical companies can drive innovation, translating computational insights into clinical advances. Furthermore, patient engagement in AI research is vital, ensuring that technologies address real-world needs and concerns.

In resume, AI has ushered in a new era for cancer research, offering tools to decipher the complexity of the disease and accelerate the development of targeted therapies. By efficiently analyzing heterogeneous data sets, identifying biomarkers, and optimizing drug discovery, AI has become an invaluable resource in the fight against cancer (Figure 1). However, to harness its full potential, challenges related to data quality, interpretability, and ethics need to be addressed. With continued innovation and collaboration, AI is poised to transform cancer research and care, bringing hope to millions of people around the world.

Conflict of interest

Amancio Carnero is an Editorial Board Member of this journal. The author declares that he has no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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REVIEW ARTICLE

The role of melanoma-derived exosomes in metastasis: Challenges and opportunities

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Abstract

Melanoma, a highly aggressive skin cancer, is characterized by its strong metastatic potential and resistance to standard treatments. Recent cancer research has emphasized the significance of exosomes, a type of extracellular vesicle and particle, in mediating cell-to-cell communication and driving tumor progression. Melanoma-derived exosomes contribute to metastasis by facilitating immune evasion, modulating the tumor microenvironment, and inducing epithelial-to-mesenchymal transition. The exosomal cargos, including nucleic acids (DNA, microRNA, long noncoding RNA, and circular RNA), proteins, lipids, and other biomolecules, play critical roles in reprogramming recipient cells to support tumor growth and spread. These exosomes also aid in forming pre-metastatic niches by transferring pro-inflammatory cytokines, extracellular matrix remodeling enzymes, and angiogenic factors to distant organs, preparing these sites for tumor colonization. Furthermore, tumor-derived exosomes promote therapy resistance by delivering drug-resistant molecular signatures, which diminish treatment efficacy. This emerging evidence highlights the therapeutic potential of exosome-based strategies, including inhibitors of exosome biogenesis and uptake or the use of engineered exosomes for targeted drug delivery. Advances in precision medicine also facilitate the use of exosome-derived molecular signatures in early-stage diagnosis and treatment monitoring through liquid biopsy. However, clinical translation remains challenging due to cargo heterogeneity, lack of standardized isolation methods, and potential off-target effects in exosome-based therapies. Addressing these challenges could lead to more effective therapies and better patient outcomes. This review synthesizes current knowledge on the biogenesis, composition, and functional roles of tumor-derived exosomes in metastasis, alongside their potential applications as biomarkers and therapeutic strategies. Deciphering exosomal dynamics in melanoma may open new avenues for advanced diagnostics and treatments.

Keywords: Melanoma; Exosome; Metastasis

1. Introduction

Melanoma is a cancer that originates from pigment-producing cells in the skin.[1] It is known for its aggressive behavior, high propensity for metastasis, and increasing incidence rates worldwide, particularly among young adults.[2] The American Cancer Society reports that melanoma is a leading cause of skin cancer-related deaths, with an estimated 99,780 new cases and 7650 fatalities in the United States in 2022.[3] The disease is classified into several types, including cutaneous melanoma, mucosal melanoma, and uveal melanoma,[4] each presenting unique challenges in terms of diagnosis, treatment, and prognosis.[5-12]

The metastatic process in melanoma is complex and involves several steps: local tissue invasion, entry into the bloodstream or lymphatic system, persistence in circulation, migration into distant tissues, and establishment at secondary sites.[13] Common sites of metastasis include nearby lymph nodes, pulmonary tissue, hepatic tissue, and the brain.[14] The prognosis for patients with metastatic melanoma remains poor, with a 5-year survival rate of <30%.[15] Although significant progress has been made with treatments such as immunotherapy and targeted therapies addressing specific genetic mutations,[16] many patients still experience disease recurrence and progression.[17]

Melanoma cells utilize key molecular pathways, such as the mitogen-activated protein kinase and phosphoinositide 3-kinase-protein kinase B signaling cascades, to drive invasive behavior and survival during metastasis.[18] In addition, immune evasion mechanisms, including the suppression of cytotoxic T-cell responses through programmed death-ligand 1 (PD-L1) expression,[19] further facilitate the metastatic cascade. During this process, melanoma-derived exosomes play a pivotal role in preparing distant organs for tumor colonization by modulating the local microenvironment. These vesicles deliver pro-inflammatory cytokines, extracellular matrix (ECM) remodeling enzymes, and angiogenic factors to distant tissues, fostering a permissive niche for tumor cell invasion.

Recent studies have emphasized the pivotal role of exosomes in cancer development, particularly in metastasis.[20-22] Exosomes, small extracellular vesicles (30 – 150 nm), originate from the endosomal membrane and are secreted into the extracellular space.[23,24] These vesicles transport biomolecules, such as proteins, lipids, and nucleic acids (DNA, long noncoding RNAs [lncRNAs], and microRNAs [miRNAs]), reflecting the molecular traits of their source cells.[25] In melanoma, exosomes actively participate in a wide range of biological processes that contribute to tumor progression and metastasis, including immune modulation, interaction with the tumor microenvironment (TME), and the facilitation of epithelial-to-mesenchymal transition (EMT).[26] The molecular cargos of melanoma-derived exosomes, such as specific DNA, miRNAs, and lncRNAs, have significant potential as indicators for early diagnosis and predictors of treatment outcomes. Furthermore, modified exosomes offer a groundbreaking solution for targeted drug administration, enabling precision medicine approaches that minimize systemic toxicity.

This review aims to provide a comprehensive summary of current knowledge regarding tumor-derived exosomes and their role in metastasis, based on literature from the National Library of Medicine, National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). We explore the biogenesis and composition of melanoma-derived exosomes, their functional implications in metastasis, and their potential as clinical diagnostics and targeted therapies. By integrating recent findings in exosome research with the broader context of melanoma biology, we aim to highlight the significance of exosomes in shaping the metastatic landscape of melanoma and identify future directions for research and clinical application.

This review covers several key aspects related to melanoma-derived exosomes: (i) the biogenesis and composition of exosomes; (ii) the cargos of exosomes, including nucleic acids, proteins, lipids, and metabolites; (iii) the functional implications of exosomes, particularly their involvement in processes such as proliferation, migration, invasion, immune modulation, and TME remodeling; and (iv) the potential use of exosomes in diagnosis and treatment, including their role as biomarkers, therapeutic targets, and drug delivery systems.

2. Exosome biogenesis and composition

Exosomes are formed through the endosomal pathway, where inward budding of the endosomal membrane generates multivesicular bodies (MVBs).[27,28] Exosomes exhibit significant heterogeneity in size, molecular composition, and biological functions, which complicates their characterization and therapeutic applications. Advanced separation techniques, such as asymmetric-flow field-flow fractionation, have identified distinct extracellular nanoparticle subpopulations, including large exosome vesicles (Exo-L, 90 – 120 nm), small exosome vesicles (Exo-S, 60 – 80 nm), and exomeres (~35 nm, non-membranous nanoparticles).[27] These subtypes carry unique proteomic, lipidomic, and transcriptomic signatures, influencing their roles in tumor progression, immune modulation, and organotropic metastasis. Traditional isolation methods, such as ultracentrifugation and size-

exclusion chromatography, often fail to distinguish these subpopulations, resulting in heterogeneous preparations. Asymmetric-flow field-flow fractionation and immunoaffinity-based separation techniques offer improved resolution, preserving vesicle integrity and specificity. Recognizing exosome heterogeneity and optimizing isolation methods are crucial for advancing biomarker discovery and therapeutic applications.[29] The endosomal sorting complexes required for transport (ESCRT) complex operates in a stepwise manner, with ESCRT-0 identifying ubiquitinated cargo, ESCRT-I and ESCRT-II facilitating membrane curvature, and ESCRT-III completing vesicle separation.[30-32] These MVBs follow two possible pathways: they can merge with lysosomes, leading to their breakdown, or be directed to the plasma membrane, where their contents are discharged into the surrounding extracellular environment as exosomes.[33] The direction of MVB trafficking is influenced by Rab GTPases, such as Rab27a and Rab27b,[34] which facilitate MVB docking and fusion with the plasma membrane, thus promoting exosome release.[35] The biogenesis process is orchestrated by multiple proteins, such as tetraspanins (cluster of differentiation [CD] 63,[36] CD81, and CD9), which are essential for cargo sorting and exosome release. The ESCRT[30] machinery is also integral to the formation of exosomes.[37]

The molecular composition of melanoma-derived exosomes is significantly distinct from that of exosomes secreted by normal cells, as they contain specific proteins, lipids, and nucleic acids that contribute to tumor progression, immune modulation, and metastasis. Tumor-derived exosomes are enriched in proteins associated with tumor progression, such as matrix metalloproteinases (MMPs), integrins, and various oncogenic signaling molecules.[38] In addition, they contain a unique profile of miRNAs that can modulate gene expression in recipient cells, influencing processes such as proliferation, invasion, and immune evasion.[39] Key examples include miR-211,[35] which downregulates the tumor suppressor brain-2 to promote invasion, and miR-155,[40] which suppresses immune cell activation by targeting the suppressor of cytokine signaling 1.

3. Exosomal cargo in melanoma

The cargo of exosomes derived from melanoma cells provides a rich source of information about the tumor's molecular landscape.

3.1. RNA

3.1.1. miRNAs

miRNAs are small RNA molecules that regulate gene expression at the post-transcriptional level. Specific miRNAs are enriched in melanoma-derived exosomes, where they modulate various aspects of tumor biology.[41,42] For instance, miR-211 is associated with the promotion of melanoma cell invasion, while miR-155 is involved in immune modulation.[43] The transfer of these miRNAs to recipient cells can alter their behavior, contributing to the metastatic process.[44,45]

3.1.2. Long non-coding RNAs

Long non-coding RNAs are non-coding RNA molecules longer than 200 nucleotides that influence gene activity through transcriptional, post-transcriptional, and epigenetic mechanisms. In melanoma, certain lncRNAs are selectively packaged into exosomes, where they contribute to tumor progression.[41] For example, lncRNA metastasis-associated lung adenocarcinoma transcript 1 plays a significant role in driving melanoma cell growth and facilitating their migratory behavior.[46] Moreover, studies reveal that metastasis-associated lung adenocarcinoma transcript 1 supports melanoma growth and metastasis by activating critical oncogenic pathways.

3.1.3. Circular RNAs (circRNAs)

circRNAs are a class of non-coding RNAs characterized by their unique covalently bonded circular configuration. They act as competitive endogenous RNAs, sequestering miRNAs and influencing the expression of downstream genes. In melanoma, circRNA cerebellar degeneration-related protein 1 (PD-1) antisense RNA has been identified as a competitive binder of miR-7-5p, which, in turn, stabilizes and upregulates oncogenes like E2F transcription factor 3.[47] This interaction promotes tumor cell proliferation, invasion, and altered glucose metabolism.[48]

3.2. DNA

Exosomal DNA (exoDNA), derived from the cell's nucleus and mitochondria, plays a crucial role in melanoma progression and metastasis. Sorting mechanisms and interactions with endosomal components are involved in its packaging.[50,51]

Studies have shown that tumor-derived exoDNA contains oncogenic mutations, which can be transferred to recipient cells, influencing TME remodeling, immune escape, and drug resistance. In addition, exoDNA serves as a biomarker for liquid biopsy,[52] offering non-invasive diagnostic potential for melanoma.[49,53]

3.3. Proteins

Tumor-derived exosomes carry key regulatory proteins, including MMPs, which degrade the ECM to promote tumor invasion and migration.[30,54] In addition, exosomes transport signaling molecules such as integrins and growth

factors, which facilitate pre-metastatic niche formation and immune modulation.[55,56] Tumor-derived exosomes also contain proteins involved in drug resistance, which modulate therapy responses by altering signaling pathways.[21]

Melanoma-derived exosomes contain a diverse set of proteins that participate in key signaling pathways associated with metastasis. For example, proteins such as MMPs facilitate ECM degradation, allowing for tumor invasion and migration.[57,58] Furthermore, exosomal lipids can modulate signaling networks and influence the activities of recipient cells, further enhancing metastatic potential.[33]

3.4. Lipids

Exosomal lipids play a significant role in melanoma progression by influencing membrane stability, intracellular signaling, and metabolic adaptation.[59] Lipid rafts in exosomes serve as platforms for signal transduction, while specific lipids, such as sphingolipids and ceramides, are involved in cell communication and apoptosis regulation.[60] Dysregulated lipid content in melanoma-derived exosomes has been associated with enhanced metastatic potential and immune evasion.[61]

3.5. Other biomolecules in exosomes

Beyond nucleic acids, proteins, and lipids, melanoma-derived exosomes also contain small metabolites that influence tumor metabolism and interactions with the microenvironment.[39] These metabolites include amino acids, sugars, and vitamins, which support tumor proliferation and modulate immune responses. Glycolytic intermediates found in tumor exosomes contribute to metabolic reprogramming, while vitamins and cofactors regulate oxidative stress and mitochondrial function.[62] Understanding the metabolic landscape of melanoma-derived exosomes could provide novel therapeutic targets for disrupting tumor-supportive pathways.[39]

4. Contribution of melanoma-derived exosomes to metastasis

Exosomes derived from melanoma cells play a multifaceted role in promoting metastasis through various mechanisms:

4.1. Promotion of tumor growth

Exosomes can carry and deliver oncogenic factors, such as growth molecules, including growth regulators and cytokines, to neighboring cells, thereby promoting tumor growth and invasion. For example, melanoma-derived exosomes contain vascular endothelial growth factor, which promotes angiogenesis and enhances tumor vascularization.[63]

4.2. Shaping the TME

Melanoma-derived exosomes are crucial in remodeling the surrounding tumor ecosystem. By transferring bioactive molecules to stromal cells, these exosomes can alter the behavior of the surrounding cells, creating a favorable environment for tumor growth and metastasis.[64] For instance, exosomes can stimulate fibroblasts to produce pro-inflammatory cytokines, leading to increased angiogenesis and enhanced nutrient supply for the tumor. Moreover, exosomes can modify the ECM, making it more conducive to tumor invasion.[65]

4.3. EMT

A key process driven by melanoma-derived exosomes is EMT,[7] a biological mechanism that enables epithelial cells to acquire migratory and invasive characteristics. Exosomal miRNAs, such as miR-211, can downregulate E-cadherin, a key adhesion molecule, promoting EMT and enhancing the invasive capacity of melanoma cells.[6] This transition not only aids in local invasion but also prepares melanoma cells for dissemination to distant organs.[26]

4.4. Immune evasion

Melanoma cells exploit exosomes to evade the immune system. Exosomes can modulate the function of immune cells, including dendritic cells and T-cells, promoting a tolerogenic environment. For instance, melanoma-derived exosomes can carry PD-L1,[66] which binds to programmed cell death PD-1 receptors on T-cells, leading to T-cell inhibition.[67] This immune suppression allows melanoma cells to escape detection and destruction, thereby facilitating their survival and proliferation.[68]

4.5. Pre-metastatic niche formation

Exosomes also contribute to the formation of pre-metastatic niches, which are sites in distant organs that are primed for the arrival and growth of metastatic cells.[64] Melanoma-derived exosomes can induce changes in the microenvironment of these distant sites, such as the lungs or liver, by promoting inflammation and altering the local cellular composition. This pre-conditioning enhances the likelihood of successful colonization by circulating melanoma cells.[69]

4.6. Biomarkers and therapeutic targets

Given their role in metastasis, melanoma-derived exosomes are being explored as potential biomarkers for disease progression and therapeutic targets.[13] The unique molecular signatures of these exosomes can provide insights into tumor dynamics and patient prognosis. Furthermore, targeting exosomal pathways – whether by inhibiting their release, blocking their uptake, or altering

their cargo – could offer a novel therapeutic strategy to combat melanoma metastasis.[70]

5. Diagnostic and therapeutic implications

The unique composition of melanoma-derived exosomes presents opportunities for their use as biomarkers and therapeutic targets.

5.1. Exosomes as biomarkers

Exosomes, which can be extracted from biological fluids such as blood, urine, and lymphatic fluid, show great promise as a resource for liquid biopsies.[71] Specific miRNAs and proteins found in exosomes have been linked to disease progression, treatment outcomes, and prognosis. For example, elevated levels of certain exosomal miRNAs[72] have been identified in advanced stages of melanoma,[73] underscoring their potential as non-invasive biomarkers for early detection and disease monitoring.[74]

5.2. Therapeutic targeting of exosomes

Targeting exosomes represents a promising therapeutic strategy in melanoma, as they contribute to tumor progression, immune modulation, and therapy resistance. Approaches to inhibit exosome release, modify their cargo, or block their uptake could disrupt tumor-promoting communication pathways. In addition, engineered exosomes offer potential for targeted drug delivery, improving therapeutic efficacy while minimizing off-target effects.[22]

However, off-target effects remain a major concern, as these therapies may affect non-target tissues, leading to unwanted side effects and reduced efficacy. Exosomes can interact with unintended cell types, potentially causing non-specific distribution and unintended interactions. The biodistribution of exogenously administered exosomes is often unpredictable, resulting in unintended accumulation in non-target organs or tissues. This non-specific distribution can lead to unwanted side effects, such as inflammation or toxicity in healthy tissues, thereby compromising overall therapeutic efficacy. These limitations are further discussed in Section 4.3, which provides a more comprehensive perspective on the challenges associated with the clinical application of exosome-based therapies.

Recent studies have identified several key exosomal proteins, signaling pathways, and RNA molecules as potential therapeutic targets in melanoma. Regulators of exosome biogenesis, cargo sorting, and uptake play crucial roles in metastasis and immune evasion. For example, Rab GTPases,[75] tetraspanins, and ESCRT-associated proteins influence exosome secretion and composition, affecting interactions with the TME.[76,77] Similarly, targeting oncogenic exosomal RNAs, such as miRNAs and long non-coding RNAs, offers a novel approach to suppress tumor growth and immune escape.[78,79]

The table below summarizes exosome-associated therapeutic targets in melanoma[80,81] (Table 1).

By targeting exosome-associated molecules and pathways, novel therapeutic strategies could limit melanoma progression, enhance immune responses, and improve drug delivery efficiency, thereby offering new directions for precision oncology.

5.3. Limitations and challenges of exosome-based therapies in melanoma

Exosome-based therapeutic strategies show great potential in the treatment of melanoma by targeting metastatic pathways and modulating the immune system. However, the clinical application of exosome-based therapies faces significant challenges. One significant obstacle is the heterogeneity of exosomal cargo, which complicates the predictability and effectiveness of treatments. Exosomes are naturally heterogeneous, carrying a wide variety of proteins, lipids, and RNAs, depending on their cell of origin and the conditions under which they are produced. This variability makes it difficult to standardize exosome preparations, which can result in inconsistent therapeutic outcomes. Differences in cargo composition can lead to unpredictable interactions with target cells, further complicating clinical applications.

Off-target effects remain a major concern for exosome-based therapies. These therapies may affect non-target tissues, leading to undesirable side effects and reduced therapeutic efficacy. Exosomes can interact with unintended cell types, potentially leading to non-specific distribution and unintended interactions. The biodistribution of exogenously administered exosomes is often unpredictable, resulting in unintended accumulation in non-target organs or tissues. This non-specific distribution can lead to side effects such as inflammation or toxicity in healthy tissues, thereby compromising the overall therapeutic efficacy. In addition, exosomes may bind to non-target receptors or cells, further increasing the risk of adverse reactions. These off-target interactions not only reduce treatment effectiveness but also pose significant safety concerns, limiting the widespread use of exosome-based therapies.

A significant challenge in the clinical translation of exosome-based therapies is the scalability of exosome production. Producing exosomes in large quantities with consistent quality and potency remains a major hurdle. The isolation and purification methods for exosomes need

Table 1. Melanoma-derived exosomes as therapeutic targets

Target	Function and role in exosomes	Therapeutic potential	References
CEMIP	Enhances exosome-mediated tumor invasion and metastasis	Inhibiting CEMIP reduces metastasis potential	22
Rab27a/b	Regulates exosome secretion and cargo release	Rab inhibitors can block tumor exosome release	75
Syntenin-1	Facilitates sorting of tumor-promoting proteins into exosomes	Suppressing syntenin-1 reduces exosomal pro-metastatic factors	76
HSP90	Involved in the exosomal transport of oncogenic signaling proteins	HSP90 inhibitors block exosomal stress responses	77
Tetraspanins (CD9, CD63, CD81)	Regulate exosome biogenesis and uptake in recipient cells	Potential for exosome-based drug delivery modifications	78
Alix	Controls ESCRT-mediated exosome formation	Alix inhibitors could disrupt melanoma exosome secretion	79
Integrins (ITGαvβ3, ITGα6β4)	Direct exosomes to specific metastatic sites	Targeting integrins can block organotropic metastasis	82
PD-L1	Delivered by tumor exosomes to suppress immune response	Anti-PD-L1 therapy enhances anti-tumor immunity	67
MicroRNAs (miR-155, miR-211)	Modulate tumor progression and immune evasion via exosomes	Blocking oncogenic miRNAs may reduce melanoma progression	83
HSP70	Promotes exosome-mediated stress adaptation	HSP70 inhibitors may impair tumor exosome function	81

Abbreviations: CD: Cluster of differentiation; CEMIP: Cell migration-inducing and hyaluronan-binding protein; ESCRT: Endosomal sorting complexes required for transport; HSP: Heat-shock protein; PD-L1: Programmed death-ligand 1.

to be optimized for clinical use to ensure both purity and consistency. Moreover, immunogenicity poses another challenge, as exosomes derived from non-autologous sources may trigger immune responses in the recipient, reducing their therapeutic efficacy. The long-term safety profile of exosome-based therapies is still not fully understood, requiring extensive research and clinical trials to assess potential risks. Furthermore, the lack of standardized protocols for exosome preparation, isolation, and characterization complicates the consistency of clinical outcomes. Clear regulatory guidelines are needed to ensure the safety and efficacy of exosome-based therapies in clinical settings.

Efficient targeted delivery of exosomes remains a critical challenge in maximizing therapeutic benefits. While exosomes hold promise for targeted delivery of therapeutic agents, their ability to reliably reach specific tissues or cells is often inconsistent. Developing strategies to enhance the specificity of exosome targeting is crucial for improving treatment outcomes and minimizing off-target effects. Advances in surface modification techniques, such as functionalizing exosomes with targeting ligands, are required to improve their specificity and efficacy. Targeting specific cell types or tissues with exosomes will be key to overcoming current limitations and ensuring that exosome-based therapies achieve their full therapeutic potential.

5.4. Emerging exosome-based therapeutic strategies

Recent advances have highlighted the therapeutic potential of engineered exosomes as both therapeutic agents and delivery systems for various diseases, including melanoma. Through genetic modification, surface engineering, and cargo loading, exosomes can be tailored to enhance targeting ability, improve drug delivery efficiency, and reduce systemic toxicity.[84] By modifying exosomal membranes or incorporating bioactive molecules, researchers aim to refine their specificity and therapeutic efficacy while minimizing off-target effects.[85]

One promising approach involves modifying exosome cargo to enhance cancer immunotherapy. Tumor-derived exosomes carrying tumor-associated antigens can stimulate antigen-presenting cells, thereby strengthening the anti-tumor immune response. In addition, engineered exosomes depleted of PD-L1, a key immune checkpoint regulator, have been explored to enhance the effectiveness of PD-1/PD-L1 inhibitors in melanoma therapy.[86] These findings indicate that engineered exosomes may complement current immunotherapies.

In the context of chemotherapy, exosomes have been modified to serve as drug carriers, improving the delivery of cytotoxic agents such as paclitaxel, doxorubicin, and cisplatin.[87] Compared to synthetic nanoparticles, exosomes

demonstrate superior biocompatibility, increased stability, and enhanced uptake by tumor cells, making them promising vehicles for precision medicine.[88] Moreover, exosome-mediated clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) gene editing has been explored as a tool to selectively disrupt oncogenic mutations, offering a novel therapeutic avenue in melanoma.[89]

Beyond oncology, engineered exosomes have shown significant promise in regenerative medicine, particularly in wound healing, neuroregeneration, and cardiac repair. Studies have demonstrated that mesenchymal stem cell-derived exosomes, enriched with growth factors such as vascular endothelial growth factor, transforming growth factor beta, and insulin-like growth factor 1, accelerate wound closure, angiogenesis, and tissue remodeling, making them attractive candidates for skin regeneration and chronic wound therapy.[90] In neurological disorders, exosomes carrying neurotrophic factors such as brain-derived neurotrophic factor and nerve growth factor have been tested for their potential to promote neuronal survival, axonal growth, and synaptic plasticity, showing therapeutic promise in conditions like Alzheimer's disease, Parkinson's disease, and spinal cord injury.[91]

Another growing application of engineered exosomes is in cardiovascular repair, where exosomes loaded with pro-angiogenic miRNAs (miR-21, miR-126, and miR-199a) have been shown to promote blood vessel formation, reduce ischemic injury, and enhance heart function following myocardial infarction.[92] These studies suggest that exosome-mediated therapy could offer a novel approach to treating ischemic heart disease and other cardiovascular disorders.

The ability to fine-tune exosomal cargos and surface properties has also enabled their use in autoimmune and inflammatory diseases. By engineering exosomes to carry anti-inflammatory cytokines (e.g., interleukin-10 and transforming growth factor-beta) or immunosuppressive RNAs, researchers have explored their application in conditions such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease.[93] These modified exosomes have shown potential in reducing inflammation, modulating immune responses, and promoting tissue repair, further expanding their therapeutic scope.

Despite these advancements, several challenges remain in exosome-based therapeutics, including scalability, reproducibility, and regulatory hurdles. The development of standardized isolation and purification techniques is crucial for ensuring the consistency and safety of exosome-based interventions. In addition, efforts to enhance exosome targeting specificity and prolong circulation time are actively being pursued to improve their therapeutic effectiveness in clinical settings.

The table below summarizes key applications of engineered exosomes in various therapeutic fields (Table 2).

5.5. Integration of exosomes with precision medicine

The unique biomarker properties of exosomes have the potential to significantly advance precision medicine,[94] particularly in the diagnosis and personalized treatment of melanoma. For example, liquid biopsy techniques that analyze exosomal miRNA expression profiles can identify patients at high risk of metastasis, providing critical insights for the early intervention. Furthermore, in the therapeutic realm, engineered exosomes can serve

Table 2. Therapeutic applications of engineered exosomes

Application	Modification strategy	Therapeutic potential	References
Cancer immunotherapy	Exosomes carrying tumor-associated antigens	Enhances antigen presentation and T-cell activation	85
Immune checkpoint blockade	PD-L1-depleted exosomes	Improves anti-PD-1 therapy efficacy	86
Chemotherapy	Drug-loaded exosomes (paclitaxel, doxorubicin, and cisplatin)	Targeted drug delivery with reduced systemic toxicity	88
Gene therapy	CRISPR/Cas9-loaded exosomes	Oncogene editing for precision cancer treatment	89
Wound healing	MSC-derived exosomes with growth factors	Accelerates tissue repair and reduces scarring	90
Neuroregeneration	Exosomes carrying BDNF, NGF, and miR-124	Supports neuronal survival and axonal repair	91
Cardiac repair	Exosomes with pro-angiogenic microRNAs (miR-21, miR-126)	Stimulates blood vessel formation and improves heart function	92
Autoimmune diseases	Immunomodulatory exosomes enriched in IL-10 and TGF-β	Reduces inflammation and modulates immune responses	93

Abbreviations: BDNF: Brain-derived neurotrophic factor; Cas9: CRISPR-associated protein 9; CRISPR: Clustered regularly interspaced short palindromic repeats; IL-10: Interleukin-10; miR: MicroRNA; MSC: Mesenchymal stem cell; NGF: Nerve growth factor; PD-L1: Programmed death-ligand 1; PD-1: Programmed cell death protein 1; TGF-β: transforming growth factor-beta.

as precise drug delivery systems, transporting anti-cancer drugs, small interfering RNAs, or CRISPR/Cas9 gene-editing tools[95] directly to tumor cells while minimizing damage to normal tissues. For instance, encapsulating chemotherapeutic agents within exosomes and delivering them to melanoma sites not only enhances therapeutic efficacy but also substantially reduces systemic toxicity. By integrating exosomal biomarker analysis with targeted delivery technologies, precision medicine holds the promise of optimizing the full spectrum of melanoma management, thereby significantly improving patient survival rates and quality of life (Figure 1).

6. Discussion

The intricate relationship between tumor-derived exosomes and metastasis underscores a significant advancement in our understanding of tumor biology and the mechanisms underlying cancer progression. Exosomes are small extracellular vesicles secreted by various cell types, including melanoma cells. They facilitate cell communication and transport a diverse array of molecular cargo, including proteins, lipids, and nucleic acids. In the context of melanoma, these vesicles play a multifaceted role in promoting metastasis by influencing the TME,[98] enhancing the invasive properties

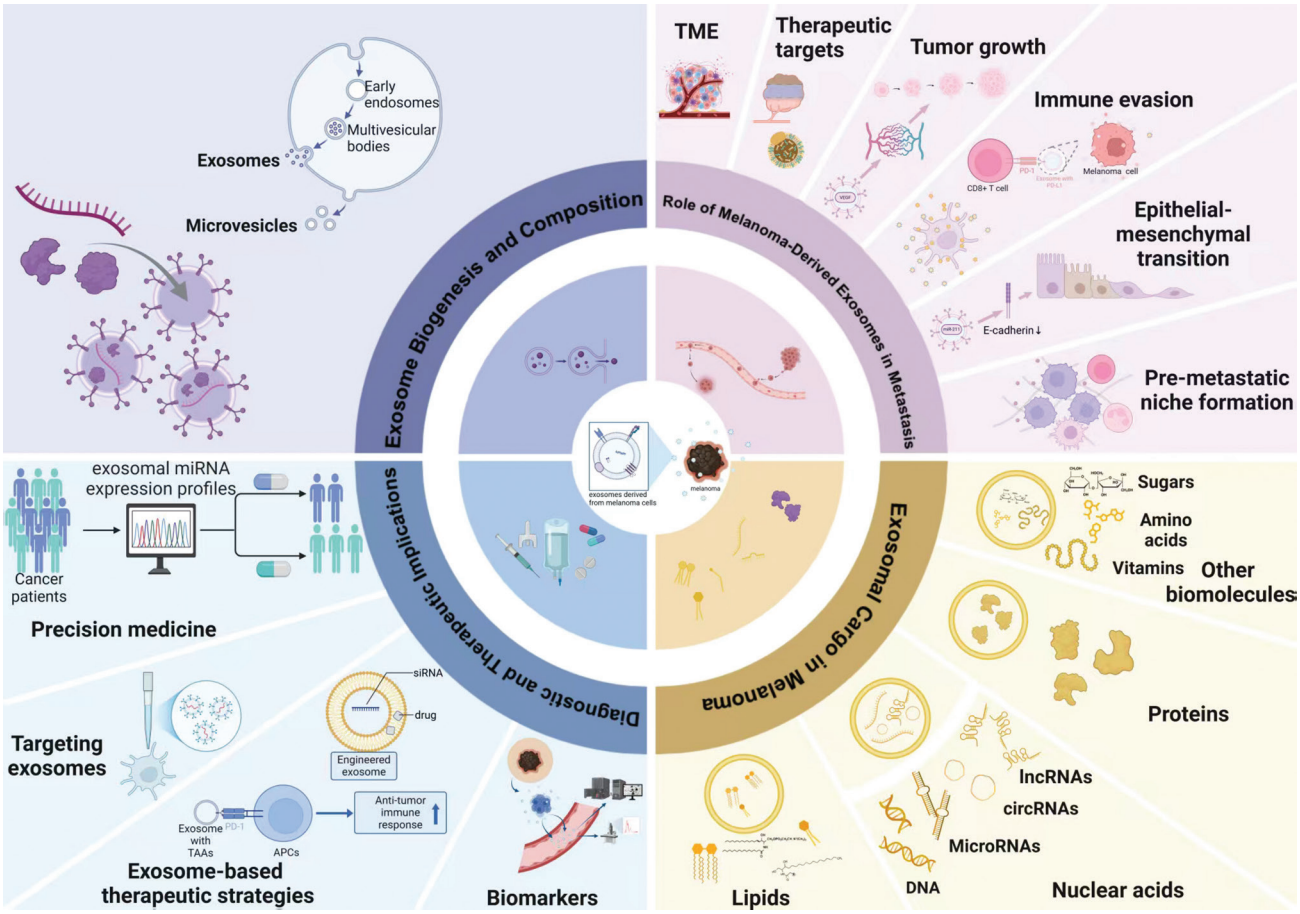

Figure 1. The multifaceted role of melanoma-derived exosomes in metastasis and therapeutic potential. This figure provides a comprehensive overview of the biogenesis, molecular composition, and functional implications of melanoma-derived exosomes. The upper-left section illustrates the process of exosome biogenesis, from their formation within MVBs to their release into the extracellular space. The lower-right section categorizes the diverse molecular cargos in exosomes into nucleic acids (miRNAs, circRNAs, lncRNAs, messenger RNAs, and DNA fragments), proteins (MMPs, integrins, heat shock proteins, and oncogenic factors), lipids (sphingolipids, cholesterol, and phospholipids), and other biomolecules (metabolites, glycoproteins, and growth factors). These components reflect the exosomes' cellular origin and mediate their pro-metastatic effects. The upper-right region highlights their critical roles in tumor metastasis, including TME[96] remodeling, immune evasion, EMT,[97] and pre-metastatic niche formation, which collectively facilitate both local and distant progression. Finally, the lower-left panel explores the diagnostic and therapeutic potential of tumor-derived exosomes, including their role as biomarkers for liquid biopsy, as well as their potential as therapeutic targets and delivery vehicles. Exosome-based strategies are being investigated for targeted drug delivery, RNA-based therapy, and immunomodulation in melanoma treatment. This integrated representation underscores the central role of exosomes in melanoma metastasis and offers a conceptual framework for future research. Image created by authors.
Abbreviations: APC: Antigen-presenting cell; CD8: Cluster of differentiation 8; miR: MicroRNA; PD-L1: Programmed death-ligand 1; PD-1: Programmed cell death protein 1; TAAs: Tumor-derived exosomes carrying tumor-associated antigens; VEGF: Vascular endothelial growth factor.

of tumor cells, and facilitating immune evasion.[39] The cargos within melanoma-derived exosomes are enriched with specific miRNAs, such as miR-211 and miR-155, which influence key pathways involved in tumor progression. For example, miR-211 can downregulate E-cadherin, a crucial adhesion molecule, promoting EMT, which, in turn, enhances tumor cell migration and invasion.[99,100] This transition is not merely isolated but is intricately linked to the TME, where exosomes can reshape surrounding stromal and immune cells, fostering a tumor-supportive niche.[101]

Moreover, tumor-derived exosomes contribute to immune cell reprogramming[102] by downregulating the expression of major histocompatibility complex molecules and promoting the development of regulatory T-cells.[103] This immune modulation is crucial for melanoma cells to escape immune surveillance, allowing for unchecked growth and metastasis.[68] The exosome-mediated transfer of bioactive molecules to distant sites further complicates the metastatic process, as exosomes establish pre-metastatic niches by modifying distant organ phenotypes, thereby enhancing melanoma cell colonization. For example, exosomal factors can induce a pro-inflammatory environment in the lungs or liver, promoting the survival and colonization of metastatic melanoma cells.[69]

Exosomes are not only promising therapeutic tools but also valuable diagnostics biomarkers. Their cargo of tumor-specific proteins and nucleic acids makes them ideal candidates for early melanoma detection and for monitoring treatment responses. Combining exosome-targeting strategies with current therapies, such as immune checkpoint inhibitors or targeted drugs, could further enhance therapeutic effectiveness by modulating the TME.

As potential biomarkers,[104] tumor-derived exosomes offer a non-invasive method for tracking disease progression and treatment response. Their unique molecular signatures provide insights into the genetic and epigenetic features of tumors, aiding in the development of personalized treatment strategies.[105] However, the clinical application of exosomes as biomarkers is hindered by challenges in standardizing isolation and characterization methods,[106] as well as validating their utility across diverse patient populations. The heterogeneity of exosomal populations and the dynamic nature of their cargo further complicate these efforts, necessitating robust methodologies to ensure reproducibility and reliability in clinical settings.[65]

Therapeutically, targeting exosomal pathways presents an innovative strategy to disrupt the communication that underpins melanoma metastasis.[107,108] Approaches such as inhibiting exosome biogenesis or blocking their uptake by recipient cells could limit the pro-metastatic effects of these vesicles. In addition, engineered exosomes are emerging as promising vehicles for targeted drug delivery, allowing for the specific delivery of therapeutic agents directly to tumor cells or the TME.[109] This strategy not only enhances the efficacy of treatments but also minimizes off-target effects, which is a significant advantage in the context of systemic therapies for melanoma.[110] Beyond inhibiting exosome biogenesis or blocking their uptake, the development of engineered exosomes offers transformative potential. Recent advancements in nanotechnology have enabled the design of exosomes with enhanced specificity, such as through surface modification with ligands or CRISPR/Cas9-mediated editing of their cargo,[111] optimizing the delivery of therapeutic agents to tumor cells or the TME. Furthermore, exosomes are increasingly recognized for their role as non-invasive biomarkers, facilitating early detection of melanoma and monitoring treatment response through liquid biopsies. Combining exosome-based therapies with immune checkpoint inhibitors or traditional treatments, such as targeted therapies and radiotherapy, could amplify therapeutic efficacy and overcome resistance mechanisms. However, challenges remain, including the need for more refined isolation techniques, mitigation of off-target effects, and a comprehensive understanding of their long-term safety profile. Addressing these hurdles through innovative research will unlock the full potential of exosomal therapies, establishing them as a cornerstone in melanoma treatment strategies. Despite significant advancements in melanoma treatment with immunotherapies and targeted therapies, several limitations remain, including off-target effects, the development of resistance to treatment, and limited effectiveness against metastatic melanoma. These challenges highlight the need for more effective and alternative treatment strategies, such as exosome-based therapies. The long-term efficacy of current therapies is often hindered by these issues, necessitating the development of strategies that can overcome these obstacles.

However, the complexity of exosomal biology demands further investigation into their biogenesis, cargo selection, and interactions within the TME. Understanding the signaling pathways that regulate exosome release and uptake will be crucial for deciphering their role in metastasis. Furthermore, research should focus on the interplay between exosomes and other components of the TME, including ECM elements and various immune cell populations, to better understand how these interactions facilitate melanoma progression.[112]

New technologies, such as next-generation sequencing and advanced imaging techniques, offer promising avenues for exploring exosomal dynamics *in vivo*.[113] Future research should validate exosomal cargo functions, their

impact on recipient cells, and the development of exosome-based therapies. By integrating exosomal research with existing melanoma treatment paradigms, we can enhance our understanding of the disease and improve patient outcomes.

The major challenges in exosome-based therapeutics stem from their heterogeneity, lack of standardized isolation and characterization methods, and regulatory barriers.[114] The diversity of exosomes, including variations in their protein, lipid, and nucleic acid cargos, poses challenges for clinical applications. This variability complicates the prediction of biological behavior and consistency in therapeutic outcomes.[37,115] Future research should focus on isolating functionally distinct exosome subpopulations to improve specificity and efficacy. The absence of standardized isolation and characterization methods limits the reproducibility and scalability of exosome-based therapies.[116] Developing robust, cost-effective, and scalable isolation techniques, alongside standardized protocols, is essential for clinical applications and inter-study comparability.[117] The clinical translation of exosome therapies also faces regulatory challenges, including concerns about safety, efficacy, and quality control. Collaboration among researchers, clinicians, and regulators is crucial for establishing clear guidelines and accelerating the approval processes.[118] Overcoming these barriers will enhance the potential of exosome-based therapies, advancing melanoma treatment and patient care.[119]

In conclusion, research on tumor-derived exosomes and their role in metastasis opens new avenues for both diagnostic and therapeutic strategies. Advancing knowledge of exosomal biology may transform melanoma management by identifying new therapeutic targets and enhancing patient outcomes.[120] The integration of exosomal research into clinical practice will require collaborative efforts across disciplines, encompassing molecular biology, immunology, and clinical oncology, to fully harness the potential of these vesicles in combating melanoma metastasis.

7. Conclusion

Melanoma-derived exosomes play a critical role in the metastatic process by influencing tumor growth, immune evasion, and the remodeling of the TME. Their unique molecular composition provides valuable insights into the biology of melanoma and offers potential avenues for diagnosis and treatment. Exosomes have shown promise as therapeutic tools due to their natural biocompatibility, ability to cross biological barriers, and potential for engineering to deliver therapeutic molecules such as RNA, proteins, or drugs. Recent studies have highlighted their utility in targeted drug delivery and enhancing immunotherapies like anti-PD-1 treatments.

However, significant challenges remain in translating these findings into clinical applications. The inherent heterogeneity of exosome cargo introduces variability in therapeutic efficacy, complicating their use as consistent treatment tools. Furthermore, the lack of standardized isolation methods and characterization protocols limits reproducibility and scalability for clinical use. Regulatory barriers, including concerns about safety, immunogenicity, and quality control, further hinder the transition from research to clinical practice.

Future research should prioritize overcoming these challenges by developing advanced technologies for isolating and characterizing exosomes, identifying functionally distinct subpopulations, and addressing regulatory requirements. By tackling these barriers, exosome-based approaches hold great potential to unlock new opportunities for effective melanoma therapies and broader applications in oncology.

Despite the promising potential of exosome-based therapies, their clinical translation faces significant barriers, including the variability of exosome cargo, the difficulty of achieving consistent isolation and purification, and regulatory challenges in developing exosome-based therapeutics. In addition, improving the targeting specificity of exosomes to reduce off-target effects and enhance their therapeutic efficacy will be crucial for the successful application of exosome-based therapies in clinical settings.

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Conflict of interest

The authors declare that they have no competing interests.

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ACCSCIENCE PUBLISHING

Tumor Discovery

REVIEW ARTICLE

Targeting glioblastoma invasion and therapy resistance: Emerging trends and molecular pathways

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Abstract

Glioblastoma (GBM) is the most common and aggressive primary central nervous system malignancy. Significant resistance to therapeutic intervention is a core feature of GBM that drives tumor recurrence and underlies the remarkably poor clinical outcomes associated with this disease. This review explores the therapeutic strategies and molecular pathways involved in GBM. Therapy resistance in GBM depends on multiple interconnected macrostructural and biomolecular mechanisms, including aggressive and diffuse invasion, tumor microtube network formation, stem-like cell enrichment, selective neurovascular permeability, an immunosuppressive microenvironment, and a high degree of inter- and intra-tumoral heterogeneity. Collectively, these pathobiological features insulate specific tumor compartments and maintain GBM viability despite significant treatment-induced cellular stress. While there is enthusiasm for addressing GBM therapeutic resistance, the scale of the challenge remains immense. The identified resistance mechanisms extensively interact and can compensate for single-target assaults. Emerging overlaps between neuro-oncology and developmental neurobiology additionally suggest that GBM may exploit therapeutic resistance mechanisms yet to be identified, functioning beyond the current scientific understanding. Thus, the scope and diversity of this problem demand a comprehensive therapeutic approach capable of targeting multiple interacting mechanisms of therapeutic resistance.

Keywords: Glioblastoma; Invasion; Tumor microtube; Cytoskeleton

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1. Introduction

Primary central nervous system (CNS) tumors are rare cancers that arise directly from cells of the brain or the spinal cord. Tumors that arise from the meningeal tissues are also categorized as primary CNS tumors, and in the United States (US), this combined category of tumors represents <1% of cancer diagnoses.[1] Still, these tumors

account for approximately 3% of US cancer deaths, and the disproportionality of this death rate is significant, considering that between 2014 and 2018, only 29.1% of primary CNS tumors were classified as malignant.[2]

Primary malignant CNS tumors arise predominantly from glial cells (80.9%) and are correspondingly called malignant gliomas.[2] Malignant gliomas develop within both the brain and spinal cord, with approximately 98% originating in the brain.[3,4] Glial cells can be categorized as astrocytes, oligodendrocytes, and ependymal cells, whereas gliomas can be categorized as astrocytomas, oligodendrogliomas, and ependymomas.

Glioblastoma (GBM) is categorized as a type of astrocytoma and is the most aggressive and common histological subtype of malignant glioma. GBM is the most diagnosed primary CNS malignancy and represents approximately half (49.1%) of diagnoses in this category of human cancer.[2] This corresponds to a US incidence rate of 3.23/100,000 people, or approximately 13,000 new GBM diagnoses each year.[2]

GBM is a rapidly fatal cancer with an incredibly poor overall survival rate. Analysis of data collected through the Surveillance, Epidemiology, and End Results Program (SEER) at the National Cancer Institute of the US National Institutes of Health (NIH) shows that the median overall survival time for GBM patients in the US was 8 months and the 5-year overall survival rate is approximately 7%[2] between 2001 and 2018. This is notably lower than the 14.6-month median overall survival time reported by Stupp *et al.*[5] SEER's inclusion of data from GBM patients who chose not to undergo treatment – for whom the median survival is reported between 1 and 3 months[6,7] – likely contributed to this statistical discrepancy. However, GBM is known for its heterogeneity,[8-10] and these data aptly highlight the persistent challenge of epidemiologically characterizing a clinical cohort that includes many poorly understood subcategories with readily observable survival differences.

The combination of low incidence and poor survival rate indicates that GBM is not a prevalent disease. At present, it is estimated that there are slightly <19,000 Americans with a documented GBM diagnosis (0.006% of the 2022 US population), and the NIH correspondingly categorizes GBM as a rare disease.[11]

The overall incidence of GBM varies greatly by age, sex, and ethnicity. GBM is known to develop at all ages, but the incidence increases with age and peaks between 75 and 79 years old for the individual sexes (Figure 1A). The average age of GBM diagnosis is 65 years old.[2] Males are 60% more likely to be diagnosed with GBM overall (incidence rate ratio = 1.6) (Figure 1B),[2] but females account for the majority of high-grade gliomas that evolve from lower-grade gliomas (previously categorized as secondary GBM).[12] GBM is more common in individuals of European ancestry,[13] and its incidence in Whites is 98% higher than in Blacks and 144% higher than in Asian/Pacific Islanders (Figure 1C).[2] Socioeconomic status (SES) is also significantly correlated with GBM incidence, and the highest SES is associated with a 45% higher GBM incidence rate than the lowest SES after controlling for self-reported race.[14]

2. GBM risk factors

GBM risk factors are poorly understood. Moderate-to-high dose ionizing radiation to the head – particularly if administered in childhood – is the only environmental risk factor known to be unequivocally associated with the development of primary CNS tumors.[15] Radiation-exposed children are more likely to develop a meningioma than a malignant glioma,[15] and this risk factor accounts for very few cases of either pathology. Other extensively studied environmental risk factors include medications, hormone exposure, diet, body habitus and body mass index, smoking, birth weight, cell phone usage, and electromagnetic field exposure.[16] None of these factors have been conclusively associated with GBM development.[16]

Heritable genetic risk factors are estimated to represent approximately 25% of overall GBM risk, but around 70% of the genetic variance underlying that risk contribution remains to be identified.[17] At present, only 5% of GBMs are observably linked with familial disease,[18] and only 1 – 4% are associated with inherited genetic disorders known to increase glioma risk.[16] Most of these disorders are associated with well-characterized loss-of-function mutations in tumor suppressor genes.[16]

3. Clinical classification of CNS tumors

The aggression, extent, and spread of systemic cancers are commonly assessed and described using the American Joint Committee on Cancer Tumor, node, metastasis (TNM) staging system.[19] In this standardized clinical model, the tumor (T) component describes the size, location, and local invasive capacity of the primary tumor.[19] The node (N) component defines the degree to which cancer infiltrates the lymph nodes that surround the primary tumor.[19] The metastasis (M) component assesses the spread of primary cancer to distant organs or tissues.[19]

The unique environment of the CNS and the correspondingly unique behavior of primary CNS tumors make the TNM staging model poorly suited to describe the behavior of GBM and other CNS cancers. For example, primary CNS tumors can be locally invasive and aggressive,

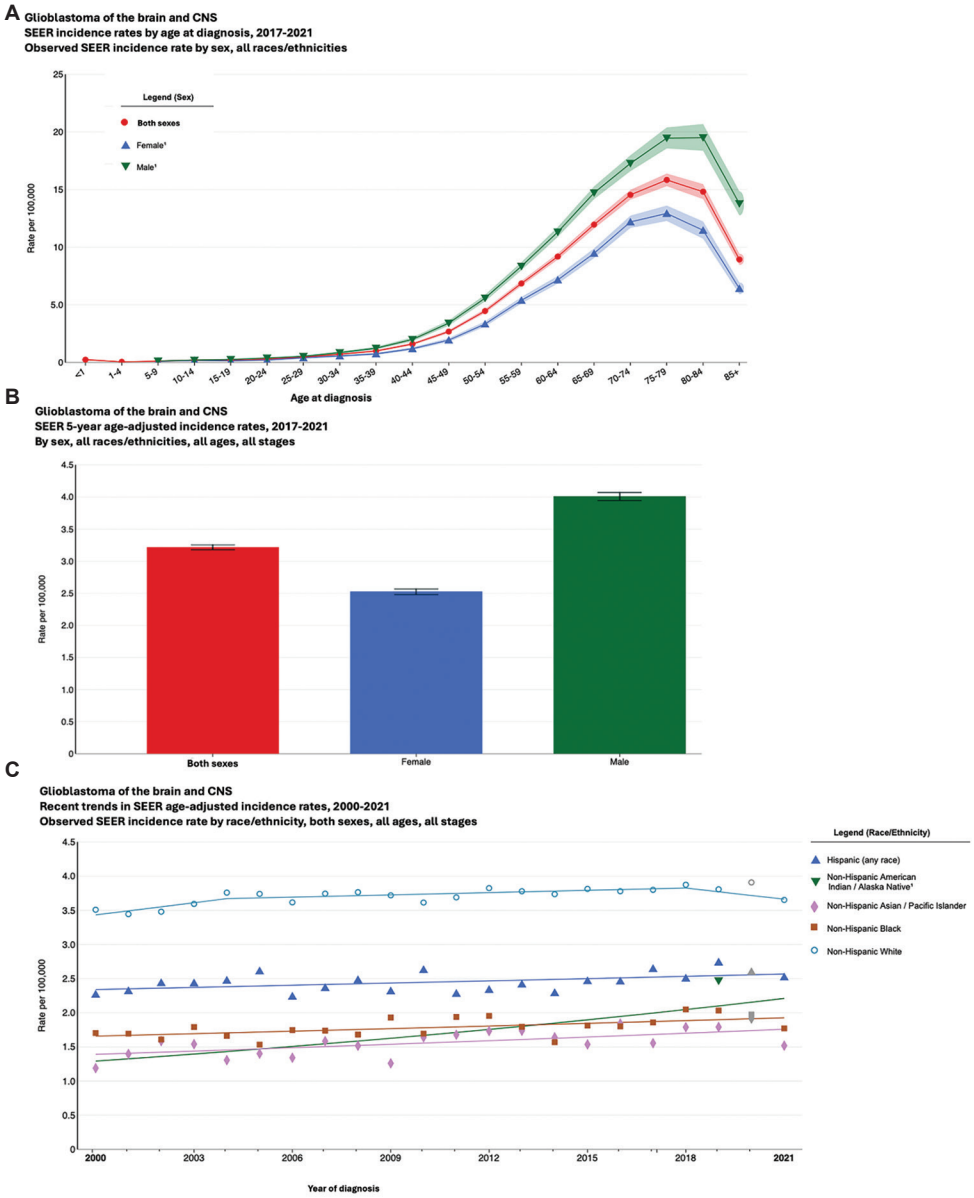

Figure 1. Overview of current GBM SEER incidence rates. (A) GBM SEER incidence rates (2017 – 2021) by age at diagnosis. (B) GBM SEER 5-year age-adjusted incidence rates (2017 – 2021) by sex. (C) GBM trends in SEER age-adjusted incidence rates (2000 – 2021) by race. Graphs generated with SEER*Explorer (Surveillance Research Program) on January 2, 2025.
Abbreviations: CNS: Central nervous system; GBM: Glioblastoma; SEER: Surveillance, Epidemiology, and End Results Program.

but they do not commonly spread into adjacent tissue types, such as the meninges or bone.[20] The CNS lymphatic system also lacks nodes and differs radically from the lymphatic organization found in systemic bodily tissues.[21] It is also extraordinarily rare for primary CNS tumors to metastasize to distant organs or tissues.[22] Collectively, these realities make TNM assessment an impractical and unhelpful tool for characterizing primary CNS cancer.

Primary CNS malignancies are assessed using the World Health Organization (WHO) Classification of Tumors of the CNS.[23] The WHO system uses a standardized grading scale (1 – 4) to characterize the behavior of these tumors from slow-growing and benign (Grade 1) to highly proliferative and aggressively malignant (Grade 4) (Table 1). GBM is classified as a WHO Grade 4 tumor.

4. GBM diagnosis

Final diagnosis and tumor grade assignment for CNS malignancies still rely on the physical evaluation of tumor tissue. Neurosurgical intervention, consisting

of a stereotactic tumor biopsy, is required to diagnose GBM definitively. Tissue is most commonly collected during standard-of-care tumor resection operations. The assignment of a GBM diagnosis was historically based on histopathological and gross tissue evaluation. Grossly, GBM is a poorly demarcated, vascularized, and friable tumor that commonly exhibits a grayish rim of rubbery tissue with a central area of yellow-tan necrosis (Figure 2A and B).[24,25] Tumors may demonstrate foci of hemorrhage and/or thrombosed vessels.[25]

Microscopically, GBM is composed of cells that resemble a spectrum of normal and reactive astrocytes that demonstrate nuclear atypia, cellular pleomorphism, mitotic activity, microvascular proliferation, and/or tissue necrosis (Figure 2C and D) (often, but not necessarily pseudopalisading necrosis).[25] Immunohistochemical evaluation for markers of astrocytic cells, such as glial fibrillary acidic protein (GFAP), or cellular proliferation, such as Ki-67, may help distinguish gliomas from other primary CNS malignancies.[24]

Molecular pathology was first incorporated into the diagnostic and grading algorithm for diffuse glioma in 2016 when the WHO released the 4th edition of CNS tumor classification guidelines.[27] In 2021, the WHO released the 5th edition of these guidelines, which strongly emphasizes integrated histomolecular diagnostics and redefines the diagnostic criteria for several categories of diffuse glioma-based molecular features.[23] Increased incorporation of diagnostic molecular markers is critically important for researchers and clinicians to better identify and stratify subgroups within the glioma and GBM diagnostic spectrum.[28] Distinct molecular subgroups of GBM have already been identified and reflect the rapidly increasing body of GBM multi-omic molecular knowledge.[29] A comprehensive summary of the histomolecular diagnostic algorithm is provided in Figure 3.

One noteworthy change featured in the 2021 WHO Guidelines is the diagnostic separation of WHO Grade 4 adult-type diffuse gliomas. Before 2021, the diagnoses "Glioblastoma, *IDH*-wild type, WHO grade 4" and "Astrocytoma, *IDH*-mutant, WHO grade 4" were both classified as GBM and referred to as primary GBM and secondary GBM, respectively. While these tumors are grossly and histologically indistinguishable, the new diagnostic algorithm reflects evidence that they are molecularly and clinically distinct malignancies.[31] They are distinguished by the mutational status of isocitrate dehydrogenases (*IDH1* and/or *IDH2*), and this is now indicated by the new diagnostic category names.[8] In gliomas, *IDH1/2* mutations cause both a loss of function and a gain of function in the IDH enzymes.[32] Distinctly,

Table 1. World Health Organization central nervous system tumor grading system

Grade	Description
Grade 1	• Slow-growing, benign
Grade 2	• Relatively slow growing • May be malignant or benign • Commonly recur as higher-grade tumors
Grade 3	• Rapid growing • Very likely to recur as higher-grade tumors • Malignant
Grade 4	• Highly proliferative • Rapid growing • Aggressively malignant

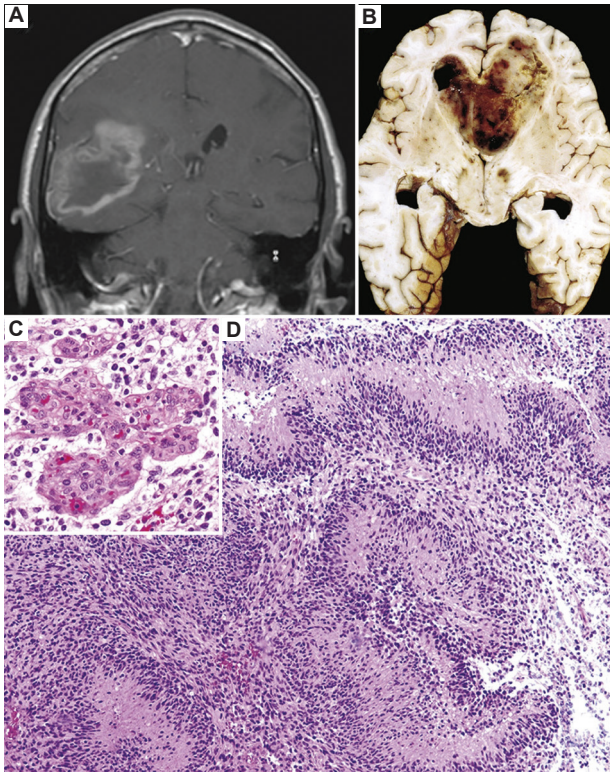

Figure 2. Glioblastoma diagnosis and histopathology. (A) Contrast T1-weighted coronal magnetic resonance image shows a large mass in the right temporal lobe with "ring" enhancement. (B) Glioblastoma appears as a necrotic, hemorrhagic, infiltrating mass. (C and D) Microscopic images of glioblastoma. Serpiginous foci of palisading necrosis (tumor nuclei lined up around the red anucleate zones of necrosis). (C) Microvascular proliferation. Image reproduced from Kumar *et al.*[26]

"Astrocytoma, *IDH*-mutant, WHO grade 4" typically (d) evolves from a pre-existing grade 2 or grade 3 astrocytoma and represents a progression of prior disease.

"Astrocytoma, *IDH*-mutant, WHO grade 4" is also associated with different clinical demographics and

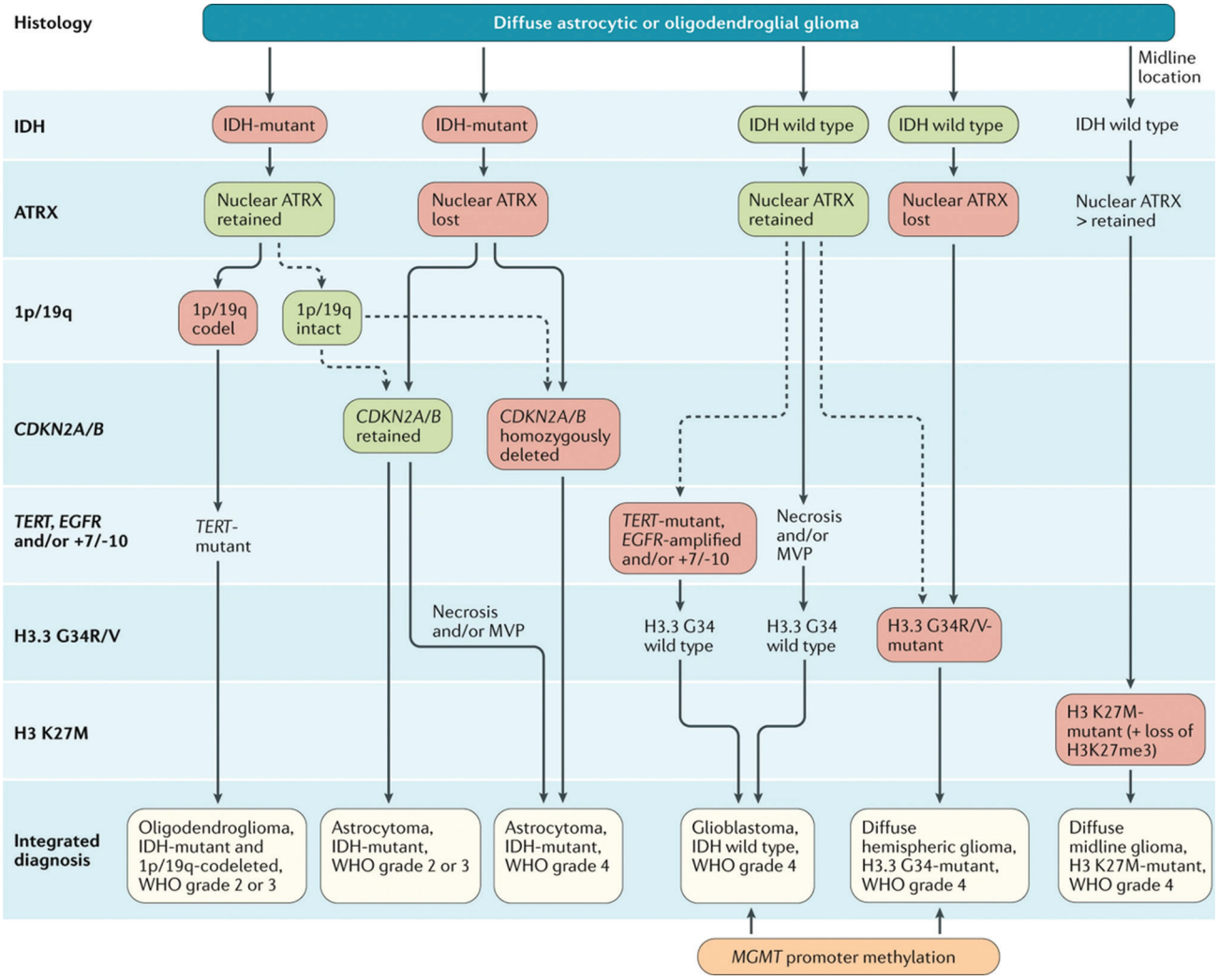

Figure 3. Molecular diagnostics of diffuse glioma in 2021. The molecular diagnostic algorithm was originally published by the European Association of Neuro-Oncology (EANO) and was subsequently adopted in the 2021 WHO central nervous system tumor guidelines. Reprinted from Weller *et al.*[30]
Abbreviations: ATRX: Alpha-thalassemia mental retardation X-linked; EGFR: Epidermal growth factor receptor; IDH: Isocitrate dehydrogenase; MGMT: O6-methylguanine-DNA methyltransferase; MVP: Microvascular proliferation; TERT: Telomerase reverse transcriptase; WHO: World Health Organization.

treatment responses. These tumors are more commonly found in females, generally affect younger adults, and are more responsive to standard-of-care chemotherapy treatment.[33] *IDH*-mutant patients also live significantly longer than *IDH*-wild type GBM patients.[8,12] Because *IDH*-wild type GBMs previously accounted for the great majority of cases in the GBM diagnostic category, the overall disease demographics were significantly skewed to reflect the primary GBM cohort. The 2021 change is intended to enable more precise epidemiological characterization of each tumor cohort, inform more accurate clinical prognostics, and facilitate efforts to develop specific therapeutic targets for each disease.

5. The clinical disease course of GBM

5.1. Clinical presentation

Adult GBMs most commonly arise above the tentorium cerebelli in the subcortical white matter of the cerebrum (frontal, temporal, parietal, and occipital lobe).[25] GBMs arising below the tentorium (cerebellum, brainstem, or spinal cord) are rare, molecularly distinct, and most prevalent in children. Initial presentation occurs with focal neurological deficits that are significantly dictated by the location in which the tumor arises. Tumors tend to be smaller upon discovery if the affected brain region causes obvious symptoms, such as vision loss, language

disruption, weakness, numbness, or pain.[34] Tumors arising in brain regions associated with more subtle symptoms, such as memory disruption, executive dysfunction, mood disturbances, or fatigue, are more frequently larger upon discovery.[34] Approximately 24% of GBM patients will present with seizures.[35] Symptoms associated with increased intracranial pressure (ICP), such as headache, nausea, sleepiness, decreased alertness, atypical pupillary response to light, and confusion, are common.[34]

Although GBM is frequently first detected with computed tomography scans in the emergent care setting, it is best visualized using magnetic resonance imaging (MRI). On MRI, GBM consistently appears as an irregularly shaped ring-enhancing mass associated with substantial peritumoral edema (Figure 2A).[36]

5.2. Standard-of-care treatment for GBM

Newly diagnosed GBMs are treated with maximally safe surgical resection, ionizing radiation, and temozolomide (TMZ) chemotherapy.[5] Among these three interventions, surgery provides the greatest survival benefit to GBM patients and resection is usually performed as soon as clinically feasible.[37] The extent of resection and residual tumor volume are both directly correlated to overall survival time.[38] The primary goal of neurosurgical GBM care is to balance aggressive tumor resection with the maintenance of patient function. Subtotal resection of GBM puts patients at considerable risk for potentially fatal intratumoral hemorrhage and cerebral edema (wounded glioma syndrome).[39] GBM resection should be pursued only when there is reasonable confidence that most of the mass can be removed. An estimated 17% of GBMs are unresectable due to their location, extent of spread, or the existence of comorbid clinical features at the time of presentation (Karnosky performance score <70).[40,41]

Once surgical wounds are healed, GBM patients complete the Stupp protocol chemoradiation. Targeted external beam radiation is typically delivered in daily 2 Gy fractions, 5 days a week, for 6 weeks (total 60 Gy) in combination with daily TMZ (75 mg/m^2).[5] Cycles of maintenance TMZ (150 – 200 mg/m^2) consisting of 5 consecutive days of treatment per 28-day cycle are then continued for approximately 6 – 12 cycles.[5] TMZ is an oral alkylating agent that creates DNA adducts at the O^6-guanine to form O^6-methylguanine.[42] O^6-methylguanine-DNA-methyltransferase (MGMT) is a DNA-damage repair response enzyme that repairs the TMZ-induced DNA adduct by removing the methyl in a degenerative reaction that depletes the MGMT enzyme pool.[42] GBMs that possess a hypermethylated *MGMT* promoter region are unable to upregulate *MGMT* transcription to regenerate the pool of functional MGMT and are thus significantly more sensitive to the effects of TMZ.[33] In 2005, Stupp *et al.* published the results of a phase III randomized multicenter clinical trial demonstrating that TMZ + radiation therapy (RT) (Stupp protocol) extends overall survival in GBM by 2.5 months in comparison to RT alone.[5,43] However, stratifying the patients enrolled in the Stupp protocol trial demonstrated that the median overall survival benefit for GBM patients with a hypermethylated *MGMT* promoter was 6.4 months. In contrast, the benefit for patients without a hypermethylated *MGMT* promoter was less than a month.[33] The Stupp protocol remains the standard-of-care for all GBM patients, regardless of MGMT methylation status, because more effective treatment alternatives do not currently exist.

Some patients additionally elect to treat their GBM with tumor-treating field (TTF) technology. TTFs are anti-mitotic medical devices that use adhesive arrays of external transducers to produce alternating electric fields of low-intensity and intermediate-frequency.[44] These alternating electric fields disrupt cell polarization and microtubule dynamics.[44] TTF clinical trials showed that the use of TTFs in combination with Stupp protocol maintenance TMZ significantly extended both the median progression-free survival and median overall survival by 2.7 and 4.9 months, respectively.[45] However, these results required that patients use the device at least 18 h/day.[45] TTF uses currently requires patients to shave their heads to adhere to transducer arrays accurately. However, adhesive-related itching is a common complaint (approximately 42%).[46]

Standard clinical care beyond the Stupp protocol for newly diagnosed GBM consists of palliative management of tumor-associated symptoms, including seizure control, pain control, and depression treatment. Some patients also pursue experimental therapy through enrollment in clinical trials. However, most clinical trials limit enrollment to cases of recurrent GBM.

5.3. Patterns of recurrence

GBM is an aggressive malignancy that exhibits significant resistance to therapeutic intervention and will recur in nearly all cases despite aggressive treatment efforts. The median progression-free survival time in GBM is 6.9 months.[5] Approximately 90% of GBMs recur within 2 cm of the surgical resection bed margin,[47,48] and this is particularly interesting considering that this is the area most heavily targeted by RT. There is no evidence indicating that tumor location, initial presentation, or other clinical factors influence the progression pattern or that progression pattern is linked to differences in overall survival time.

There is currently no standard-of-care treatment protocol for recurrent GBM. Repeat surgical resection is sometimes appropriate, though patient quality of life must be considered alongside the potential survival benefits provided by a second operation. Patients commonly pursue treatment with bevacizumab – an anti-vascular endothelial growth factor monoclonal antibody – but it has not been shown to increase overall survival and may instead increase invasive infiltration of the brain.[49,50] Other commonly pursued treatment options at the time of recurrence include carmustine and experimental therapies through clinical trial enrollment.

5.4. Cause of death

The end-of-life phase of GBM is characterized by a significant progression of neurological deficits. The majority of GBM patients exhibit decreased consciousness, dysphagia, and fever within the last days of life.[51,52] Seizures, headaches, and incontinence are also common.[53,54] Terminal-phase GBM patients report significantly lower rates of bodily pain than other cancer patients,[51] but much higher rates of depression[55] and cognitive dysfunction.[56] Causes of death routinely reported in GBM patients are associated with increased ICP and diffuse tumor infiltration of the brain and brainstem and include brain herniation, seizure, tumor-related brain hemorrhage, and dyspnea or respiratory arrest.[57,58]

6. Roles of the cytoskeleton in GBM

The cytoskeleton is an organized and dynamic meshwork of protein filaments that reinforce cell membranes, provide cells with shape/structural integrity, and facilitate essential cellular functions. In eukaryotic cells, the cytoskeleton is composed of three classes of filamentous fibers: actin, intermediate filaments, and microtubules.

Actin is a ubiquitous and evolutionarily conserved protein that makes up approximately 15% of the total protein in human cells.[59] The actin cytoskeletal system is composed of monomeric or globular actin (G-actin) and filamentous actin (F-actin). F-actin forms through the polymerization of G-actin monomers into a polarized, double-stranded helical fiber.[60] Actin filaments are highly concentrated at the cell periphery and form organized arrays of bundles and networks that underlie and give structure to the plasma membrane.[61] Manipulation of the balance between F-actin polymerization/depolymerization underlies the generation of different types of membrane-deforming forces required for cellular motility and many other essential homeostatic processes, such as endocytosis and cytokinesis.

Microtubules are long polarized fibers.[62] Like all cytoskeletal filaments, microtubules are composed of arrays of monomeric protein building blocks that organize into higher-order filamentous polymers.[63] The fundamental protein unit of microtubules is tubulin. Microtubules also cycle through phases of gradual polymerization and rapid depolymerization at the plus end.[63] A large cohort of microtubule-binding proteins regulates microtubule dynamics through a diverse set of functions, such as nucleation, capping, trafficking, stabilizing, destabilizing, bundling, cross-linking, and integration with other cytoskeletal elements.[63]

Microtubules participate in a wide variety of cellular processes. They form portions of the mitotic spindle apparatus that separate sister chromosomes.[64] They are scaffolds for motor-driven cellular trafficking, and their ultrastructural organization within the cell plays a large role in determining the distribution of other intracellular organelles.[65] This makes microtubule organization extremely important for cells that require polarity to execute physiologic functions (i.e., neurons and brush border epithelia), as a loss of microtubule organization leads to a profound disruption in internal cellular organization.[65] Microtubules are important mediators of cellular motility, and this is especially true in neurons and astrocytes where lamellipodial extension is limited, and microtubules closely underlie the leading edge membranes of migrating cells and developing neuritis.[66]

The most genetically diverse class of cytoskeletal fibers is the intermediate filaments, which are a family of cytoskeletal fibers derived from 73 different genes.[67] Despite substantial genetic diversity, intermediate filaments are remarkably consistent in size.[68,69] Intermediate filaments exhibit a high degree of homology in their sequences and structures.[70]

Intermediate filament expression profile varies greatly by cell and tissue type. For example, keratins are highly expressed in epithelial cells, whereas mesenchymal cells more commonly express vimentin.[67] The intermediate filaments GFAP and Nestin are particularly relevant to GBM biology. GFAP is used as a glial cell marker in GBM diagnostics,[24] and Nestin is a well-established marker of CNS neural progenitor cells that are also detected in GBM stem-like cells (GBMSC).[71]

7. GBM invasion and the cytoskeleton

7.1. Invasion patterns in GBM

Macroscopically, GBM favors invasion along pre-existing CNS structures, such as the white matter tracts (i.e., corpus callosum) and blood vessels (i.e., perivascular space/Virchow Robin Space).[72] Migration within the subarachnoid/leptomeningeal space, which is continuous

with the perivascular space, occurs but is less common.[72] GBM does not invade across sulcal margins.[72]

Both white-matter tracts and blood vessel scaffolds are invaded by GBM cells.[72] Signaling pathways underlying white-matter tract invasion remain largely unknown,[73] while molecular drivers of perivascular GBM invasion are well-characterized.[74] Endothelial basement membranes are enriched with collagen and fibronectin,[75] providing valuable substrates for integrin engagement that facilitate coordinated movement.[76] Blood vessels offer a large supply of oxygen/nutrients that support malignant proliferation and attract invasive cells. In contrast, the parenchymal extracellular matrix (ECM) lacks stiffening molecules such as collagen and fibronectin and is instead composed of hygroscopic molecules such as proteoglycans, hyaluronans, tenascins, and link proteins.[77] These molecules retain water, creating a gelatinous ECM that densely fills the extracellular compartment and leaves a limited amount of unoccupied extracellular space available to accommodate invasive movement.[78] Intraparenchymal invasion requires GBM cells to erode their way through the existing tissue architecture.[79] Still, GBM extensively invades cerebral gray matter, using proteolytic-guided mesenchymal and collective invasion strategies.[80-82]

7.2. Molecular drivers of GBM invasion: Rho proteins and their effectors

Dynamic remodeling of both the actin and microtubule cytoskeletal systems is required for microstructural and macrostructural patterns of GBM invasion.[83] Actin polymerization drives leading-edge protrusions, and genetic or pharmaceutical targeting of actin nucleator proteins, such as formins or Arp2/3, have been shown to reduce GBM invasion.[84-89] Microtubules are also required to support elongating cell protrusions and to transport ECM-degrading enzymes to leading-edge structures.[90,91] Microtubule targeting agents also reduce GBM invasion.[91,92]

7.2.1. Rho guanosine triphosphatases (GTPases) in GBM invasion

In GBM, differential activation of molecular switches called Rho GTPases coordinates cytoskeletal remodeling required for invasive motility[93] and dictates specific invasion programs.[94] Rho GTPases cell division control protein 42 homolog (Cdc42) and Ras-related C3 botulinum toxin substrate 1 (Rac1) are upregulated in gliomas relative to normal brain tissue.[95] Cdc42 and Rac1 activation is associated with pseudopodial extension into the brain parenchyma and guidance of other tumor cells with lower Cdc42 and Rac1 activation toward the invasive front.[94] In U87 GBM spheroid invasion, activated Cdc42 increased migration and invasion, whereas Cdc42 depletion reduced invasion.[96] Rac1 inhibition suppressed GBM cell invasion.[97]

RhoA's role in the GBM invasion is less well-defined. RhoA expression decreases with increasing grade of glial malignancy.[98] RhoA and Rac1 are known to be functionally antagonistic, with Rac1 activation predominating in mesenchymal migration and RhoA mediating the contractility required for so-called amoeboid motility.[99] Some RhoA activity is required for early adhesion and trailing edge contraction in mesenchymal motility, and RhoA regulates the expression of transmembrane matrix metalloproteases (MMPs) that remodel the ECM for mesenchymal invasion.[100] *In vivo*, RhoA activation is predominantly associated with perivascular invasion.[94] Importantly, directed invasion in GBM can be triggered in response to ECM composition (stiffness, porosity, and topography),[101] autocrine or paracrine signaling factors (pleiotrophin, CXCL12, glutamate),[102-104] or ion flux (Ca^{2+}, Cl^-, K^+).[105-107]

7.2.2. Roles for GTPases effector proteins in GBM invasion: The formins

The formin family of proteins is an evolutionarily conserved group of Rho GTPase effector proteins that make essential contributions to the organization and maintenance of the actin and microtubule cytoskeletal systems.[108] Human cells express at least 15 different formin family proteins. The diaphanous-related formin subfamily, also referred to as mammalian diaphanous-related formins (mDias), is the most extensively characterized subgroup of formins and includes mDia1, mDia2, and mDia3 (Figure 4).

The mDia formins are well known for their roles in facilitating actin polymerization. The exposed formin homology (FH2) domains of active mDia formins homodimerize to form a ring structure that stabilizes G-actin trimers and catalyzes energetically unfavorable nucleation of actin filaments. mDia formins are among the few Rho GTPase effector proteins that directly influence both actin and microtubule dynamics.[110] In addition to their roles as mediators of actin nucleation, polymerization, and bundling, mDias directly bind to and stabilize microtubules.[111,112]

Temporal regulation of mDias is controlled by Rho GTPase activity, which is, in turn, controlled by external signaling cues. Thus, Rho GTPases link mDia activation with the environmentally directed cellular demand for cytoskeletal modification. Several different Rho GTPases activate mDia1, mDia2, and mDia3, with each isoform exhibiting different GTPase specificities that facilitate a widely diverse set of cellular processes and macrostructural modifications.

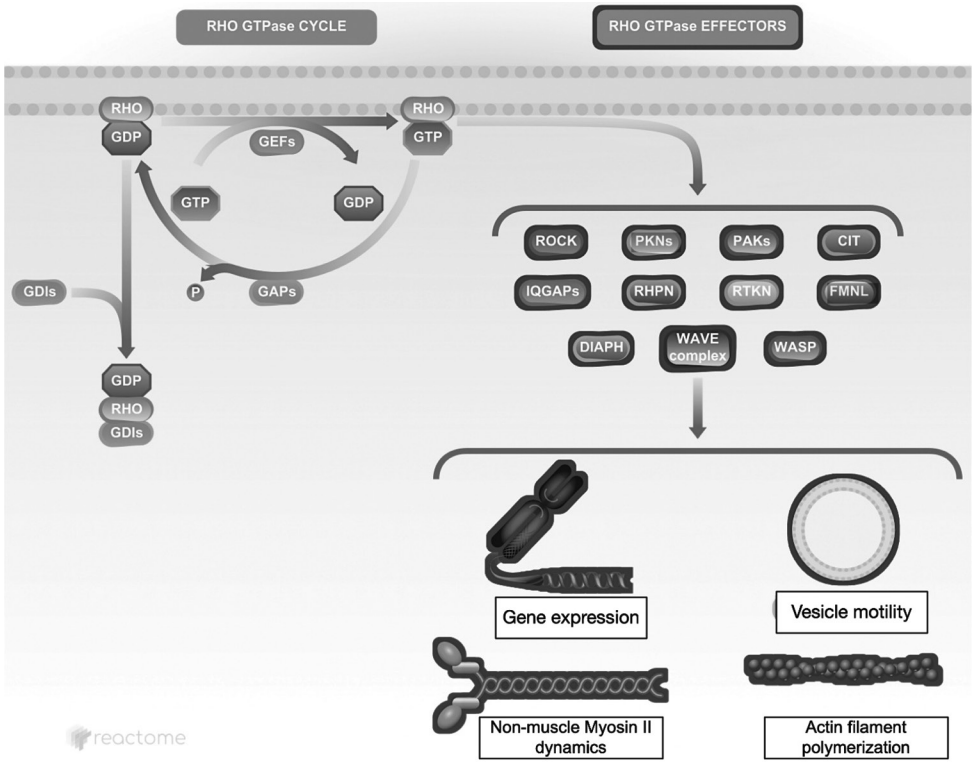

Figure 4. Overview of Rho guanosine triphosphatases (GTPases) and mammalian diaphanous (mDia) formins interplay. The Rho GTPases are molecular switches that influence gene expression, vesicle motility, non-muscle myosin II dynamics, and actin filament polymerization through association with effector proteins, including mDia proteins, which are encoded by the DIAPH genes. Reproduced from Reactome.[109]

The distinct morphology and complex physiology of CNS cells critically rely on the organization and adaptability of the cytoskeleton. Thus, mDia formins directly participate in multiple neurodevelopmental processes, and their mutations are associated with neurodevelopmental defects. Within the mDia subfamily, mDia1's contributions to neurodevelopment are well understood. mDia1 is found in the perinuclear region and co-localizes with centrosomes and mitotic spindles in cortical neural progenitor cells.[113] Multiple brain regions continue to express high levels of *DIAPH1* (the gene encoding mDia1) throughout development and adulthood.[114-116] mDia1 also supports stromal cell-derived factor-α chemoattractant-mediated axon formation in entorhinal cortical neurons[117] and cerebellar granule cells,[114] and is required for normal dendrite formation[118,119] and adherens junction formation in the ventricular zone.[115]

The mDia formins contribute significantly to GBM pathophysiology. In C6 rat glioma cells, mDia1 knockdown blocked directed migration, prevented focal adhesion turnover, and impaired the localization of Cdc42 and Rac1 at the leading edge of invasive cells.[85] Gliomas express higher levels of mDia1 relative to normal brain tissue.[86,120] In U87 glioma cells, mDia1 knockdown impaired tumor cell proliferation both *in vitro* and *in vivo* and increased tumor cell apoptosis.[86] Decreased mDia1 expression also reduced invasion and invadopodia formation[86] and downregulated the expression and activity of ECM-degrading MMPs (MMP2 and MMP9).[120] It was also demonstrated that mDia formins can be targeted with small-molecule compounds in GBM and that both broadly activating and inhibiting mDias decrease GBM invasion.[84] Agonism of mDia formins disrupts the structural viability of pro-invasive GBM structures called tumor microtubes (TMs),[91] and this phenotype was mirrored in mDia2 knockdown experiments.[87] mDia2 contributes to the maintenance of the GBM stem cell phenotype by critically participating in the Wiskott–Aldrich syndrome protein-driven stabilization of YAP/TAZ signaling.[121] Other studies have also shown that several subfamilies of formins may participate in GBM invasion.[87-89,122]

Many essential cellular functions require the activity of mDia formins to be finely tuned through both time and cellular space. Correspondingly, any disruption to mDia function or regulation threatens cellular homeostasis and multiple mDia-targeting strategies cause cytotoxicity that could be therapeutically exploited.

7.3. Therapy resistance and other clinical consequences of GBM invasion

Aggressive and diffuse invasion is a significant contributor to poor survival in GBM. Diffuse invasion creates tumors with poorly defined margins that are impossible to resect completely with surgery. Early drastic attempts to remove the entire affected brain hemisphere failed to prevent tumor recurrence on the contralateral side.[123] Thus, while GBM initially presents as a distinguishable mass, diffuse invasion fundamentally makes GBM a whole-brain disease.[124] GBM cells invading the blood vessels also strip the astrocytic end feet from the endothelial basement membrane and secrete enzymes that damage endothelial tight junctions and degrade the basal lamina.[82,125]

This invasive denuding and degrading of the endothelium triggers reactive gliosis and disrupts the neurovascular unit, thereby initiating a pathological cascade that includes the loss of blood-brain barrier (BBB) integrity, loss of activity-dependent blood flow regulation, serum leakage, and uncontrolled CNS access for ions, toxic and inflammatory molecules, and immune cells, impaired CNS uptake of glucose and oxygen, hypoxia, necrosis, and edema.[82,125,126]

In contrast, intraparenchymal invasion carries cells far beyond the margin of the radiation target and into areas of the brain with a robustly intact BBB, where they are largely inaccessible to immune cells and therapeutic drugs.[127] Some evidence additionally suggests that invasive astrocytes may limit cell cycle progression and could, therefore, be less sensitive to conventional therapies that generally target proliferating cells.[128-130] Proteolysis and glutamate-mediated intraparenchymal invasion also physically erode the normal neural architecture, thereby disrupting circuit control, triggering seizures, and ultimately leading to functional deterioration.[131,132]

In addition, many glioma therapies are known to exacerbate the invasive motility that drives tumor recurrence and neurological decline.[49,50,133,134] Most GBMs exhibit invasive behavior and significant therapy resistance at the time of tumor recurrence. Thus, invasive motility remains a primary obstacle to the successful treatment of GBM.[135]

8. GBM TMs

8.1. TM structure and morphology

TMs are invasive neurite-like protrusions that extend from the cell bodies of diffuse astrocytoma cells into the surrounding brain parenchyma.[136] TMs are structurally enriched with actin and microtubules, but they locally express myosin IIa, protein disulfide isomerase, β-catenin, β-parvin, GFAP, Nestin, connexin43 (Cx43), growth-associated-protein 43 (GAP-43), and tweety-homolog 1 (TTYH1).[136,137] Although they share many features with other protrusive cellular structures, such as invadopodia and tunneling nanotubes (which are also found in GBM),[138] TMs are morphologically distinguished by their remarkable length and capacity for long-term stability.[136] Bona fide TMs are at least 50 μm long and have an average cross-sectional area of approximately 1.5 μm^2.[136] TMs have been observed to exceed 500 μm *in vivo*[136] and commonly surpass 1000 μm in *in vitro* models (Figure 5). TMs also exhibit significant plasticity in their temporal stability. They may be stable for weeks to months or dynamically remodeled to drive invasion at the tumor-brain interface.[136]

8.2. TM networks

TMs frequently arborize and interconnect into a multicellular network. Cx43-mediated gap junctions are evident at TM cross points and enable the TM network to function as a syncytium.[136] TM networks bi-directionally propagate intercellular calcium waves (ICWs) similar to those observed in the neurodevelopmental migration of neural progenitor cells[136,139] (Figure 6). TM networks also exchange signaling molecules[140] and traffic organelles, including mitochondria.[136] TM length and quantity increase with increasing astrocytoma grade, but they are not regularly observed in 1p/19q co-deleted oligodendrogliomas,[136] which may partially explain the therapeutic response and survival difference between the astrocytoma and oligodendroglioma cohorts.

8.3. Molecular understanding of TMs

Molecular drivers of TM formation have thus far been predominantly identified through *in silico* comparison

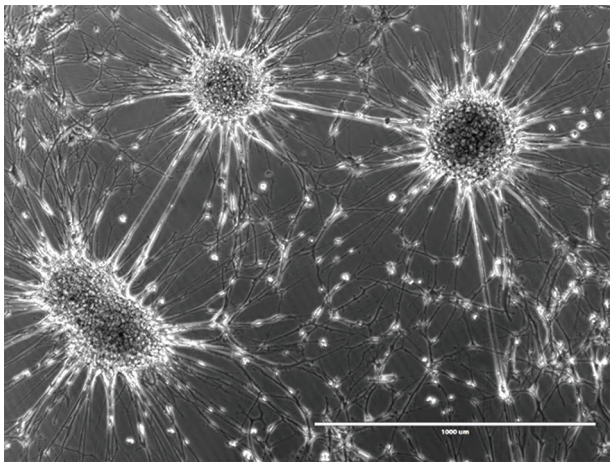

Figure 5. Tumor microtubes *in vitro*. Phase contrast image of three-dimensional networked patient-derived cell cultures demonstrates neurospheres and individually invasive cells highly connected through tumor microtubes. Scale bar = 1000 μm. Image created by the author(s).

of The Cancer Genome Atlas (*TCGA*) gene sets from 1p/19q co-deleted gliomas (i.e., oligodendrogliomas/TM-poor) and 1p/19q non-co-deleted gliomas (i.e., astrocytomas/TM-rich).[136,137] These efforts implicated pathways associated with normal neurodevelopmental processes. Pathways associated with stemness and underlying neurite outgrowth, such as signaling of the Rho family of GTPases, integrins, phospholipase C, and neurotrophin and tropomyosin/tyrosine receptor kinase signaling, are upregulated in TM-connected cells compared to unconnected controls.[71,136] Knockdown studies subsequently confirmed the roles of GAP-43,[136] TTYH1,[137] and thrombospondin 1 in TM formation.[141]

8.4. TMs in GBM invasion

At the invasive front, TMs act as dynamic probes that extend and retract in response to environmental cues. Established TMs act as scaffolds facilitating the movement of GBM nuclei into the brain parenchyma.[136] TMs display at least two phenotypes, wherein one, they are highly dynamic and associated with GBM cells exhibiting one to two minimally arborized TMs that drive invasive motility.[137] In the other phenotype, TMs are remarkably stable and associated with stationary GBM cells exhibiting four or more TMs that extensively arborize and connect to an extensive TM network.[137] Experimental knockdown of TTYH1 strongly inhibited the formation of invasive TMs, whereas networked TMs were largely unaffected.[137] Despite this, TTYH1 knockdown was associated with a significant survival advantage in a patient-derived xenograft (PDX) model of GBMSCs,[137] suggesting that targeting TM-associated invasion is a promising therapeutic strategy in GBM.[91,92]

8.5. TMs and the neuron-glioma synapse (NGS)

TMs receive synaptic input from normal neurons via a one-way, glutamatergic/alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated transmission. These NGSs generate spontaneous excitatory post-synaptic currents and slow inward currents that induce ICWs within TM networks that support the growth, invasion, and excitotoxic impact of GBM cells in the brain.[106,107] Treatment with the Food and Drug Administration-approved AMPAR antagonist perampanel reduced the invasion and growth of tumor cells and increased overall survival in PDX models of both adult and pediatric high-grade glioma.[106,143] Perampanel is well-tolerated, safe, and effective at reducing seizures in glioma-associated epilepsy.[144] In a 2009 trial, the pre-clinical AMPAR antagonist talampanel extended median overall survival from 14.6 to 20.3 months in patients with newly diagnosed GBM.[145] Tumor volume was also reduced in 8/10 glioma patients treated with six months of perampanel for glioma-associated epilepsy.[146] Targeting NGS activity with AMPAR antagonists is, therefore, a viable and promising therapeutic strategy in GBM. However, the use of perampanel has not yet been evaluated in clinical trials.

8.6. TMs in GBM therapy resistance

Tumor microtubule networks facilitate resistance to all three components of the GBM standard-of-care therapy (Figure 7). The cellular cohort that survives radiation and TMZ treatment is overwhelmingly comprised of TM-connected cells.[136,147] The TM network's ability to use ICWs to maintain calcium homeostasis within connected

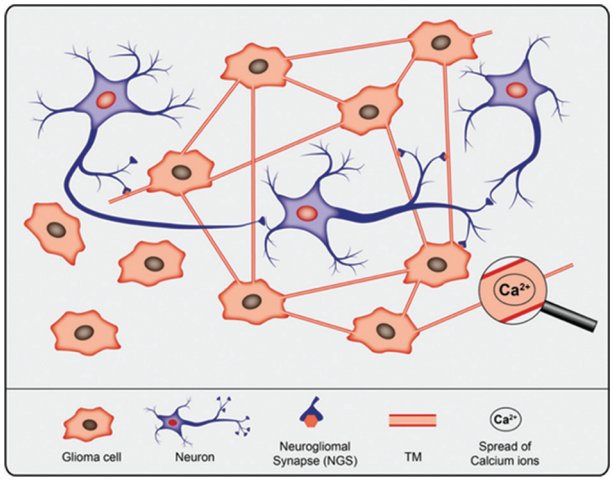

Figure 6. Schematic illustration of the gliomal network, the neuronal network, and their interconnectivity. Reproduced from Roehlecke *et al.*[142]

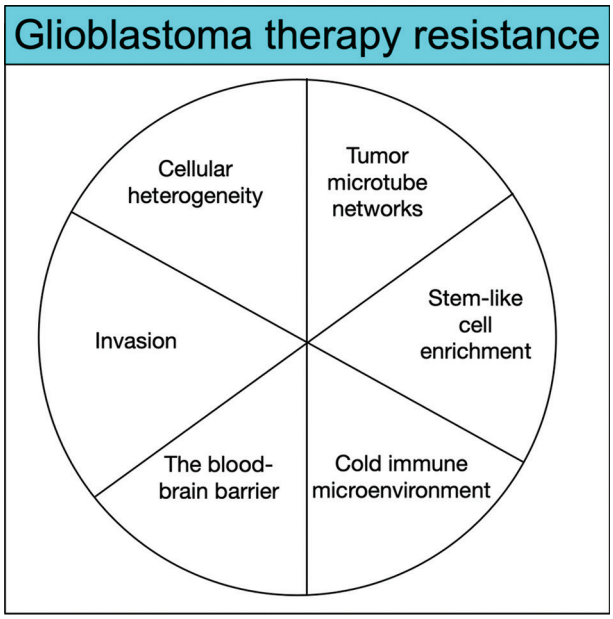

Figure 7. Multiple components of glioblastoma resistance

cells likely contributes to the observed survival advantage, as radiotherapy-induced cellular toxicity requires increases in intracellular calcium levels.[148] TM-connected cells extend new TMs toward the surgical resection lesions and into the resection bed within 3 days of tumor debulking surgery.[147] Following resection, the increase in tumor cell density within the lesion significantly outpaced the increase in tumor cell density in perilesional and distant brain tissue, and the invasive velocity was significantly directed toward the lesion.[147] GAP-43 deficient tumors did not robustly polymerize TMs toward the resection cavity and exhibited similar lesional density increases, suggesting that TMs may facilitate the coordinated repopulation of surgical lesions. This may explain why GBMs so commonly recur within 2 cm of the resection cavity margin despite aggressive regional radiation targeting.[147] Genetic targeting of TM networks in GBM PDX models decreased tumor burden, tumor invasion, and repopulation of surgical resection cavities while significantly increasing overall survival.[136,147] TMs serve as an excellent example of the ways in which the characterization of GBM pathophysiology is increasingly intersecting with developmental neuroscience.

9. Additional mechanisms of GBM therapy resistance

9.1. The BBB

The BBB is a physical and molecular feature of the CNS microvasculature that significantly prevents circulating ions, molecules, and cells from entering CNS tissue. Physically, CNS capillary beds are non-fenestrated, with endothelial cells that form continuous tight junctions.[149] This blocks paracellular solute diffusion and forces all agents to cross through endothelial cells to gain access to the CNS.[150] However, passage through CNS endothelial cells is also biochemically limited. CNS endothelial cells do not readily pinocytose molecules, and transcellular solute movement is, therefore, very difficult.[151] CNS endothelial cells additionally express luminal efflux pumps that actively export the majority of molecules that successfully pass through the endothelial membrane.[152] The barrier functions of CNS endothelial cells are further reinforced by (i) the inner vascular basement membrane and the outer parenchymal basement membrane (glial limitans),[151] (ii) pericytes, which are embedded in the vascular basement membrane and control the diameter of capillary vessels, regulate angiogenesis, deposit ECM, and control CNS immune cell trafficking,[153,154] and (iii) astrocytic end feet that link neuronal activity with blood vessel diameter and flow.[155] In the normal brain, these features collectively make up the neurovascular unit and block approximately 98% of blood-soluble molecules from accessing the CNS.[156]

The selectivity of the BBB significantly limits the number and diversity of therapeutic agents that can be used to treat CNS pathology, including GBM. While the GBM disease process notoriously disrupts the BBB, this breakdown is far from being homogenous or complete.[150] Imaging is regularly used in GBM care to identify areas of BBB breakdown, which enhances upon administration of a gadolinium contrast agent. However, many studies demonstrate that the GBM tumor burden extends far beyond this arbitrary imaging margin and into areas that remain sheltered by an intact BBB.[150] In addition, GBM causes BBB to be permeable by destroying the integrity of the neuro vasculature, not by enhancing controlled diffusion mechanisms.[82] The resultant indiscriminate leakage of fluid and ions through compromised vessels elevates the tissue oncotic pressure and causes edema that physically resists intratumoral drug penetration.[157] The BBB remains a significant hindrance to effective GBM treatment. Hence, developing strategies to circumvent this barrier is an area of concentrated research effort.

9.2. The immune-cold GBM microenvironment

The physical confines of the cranial vault significantly limit the amount of inflammation (and associated swelling) that the CNS can accommodate while still maintaining physiologic ICP. This mechanism underlies the evolution of existing anatomic and cellular mechanisms that tightly control immune access and function within the CNS.[158] Anatomically, the BBB significantly restricts circulating immune cells from entering the CNS tissue. While a limited number of peripheral immune cells are capable of entering the CNS under healthy conditions, baseline immune surveillance within the CNS is predominantly executed by resident microglia, which account for approximately 10% of CNS cells.[159] This reality notably limits not only inherent anti-GBM immune activity but also anti-GBM immunotherapeutics.[158]

CNS cells also use molecular mechanisms to limit intra-axial inflammation. The normal brain basally expresses and secretes high levels of the anti-inflammatory cytokines transforming growth factor-β and interleukin-10, which are further upregulated in the context of intracranial tumor development.[160] Microglia and infiltrating macrophages make up the majority of brain tumor-associated immune cells.[161] In GBM, tumor-associated macrophages predominantly exhibit an M1/pro-tumor phenotype that poorly activates the T-cell response.[162,163] Glioma cells also actively recruit regulatory T-cells and suppress T-cell activity by secreting indolamine 2,3-dioxygenase.[164,165] Thus, the population of tumor-infiltrating lymphocytes that do successfully infiltrate the tumor commonly exhibit an exhausted phenotype.[166]

Furthermore, intracranial tumors can cause T-cells to be sequestered within the bone marrow, and significant portions of GBM patients exhibit profound systemic baseline lymphopenia that can be further exacerbated by treatment.[167,168] Thus far, attempts to target GBM's intrinsic immunosuppressive mechanisms therapeutically have been largely unsuccessful.[158] Importantly, efforts to develop new immunotherapies in GBM must carefully balance increases in tumor-associated immune activity with the need to maintain physiologic ICP.[158]

9.3. Cellular heterogeneity

GBM also exhibits significant intratumoral heterogeneity that is attributable to genetic, epigenetic, developmental, and microenvironmental variation. Single-cell RNA sequencing analyses demonstrate that mixed populations of classical, mesenchymal, and pro-neural cells routinely co-exist within individual GBM tumors and that predominating TCGA subtype can vary significantly between different regions of a single tumor.[169] Each TCGA subtype is associated with a different tumor microenvironment immune signature.[170] Individual GBMs also harbor at least four different highly plastic neural-progenitor-like cellular states that interconvert in response to microenvironmental changes.[10] Remarkably, xenografts exclusively seeded from any single progenitor cell state consistently generate tumors comprised of all four signatures.[10]

The clinical targetability of identified GBM diversity remains unrealized, but it is well-recognized that clonal diversity and plasticity contribute significantly to GBM therapy resistance. Cancer therapies provide selective pressure that ultimately enhances the outgrowth of treatment-resistant clones.[171] In addition, both TMZ and RT are mutagenic treatments that can cause mutations that further drive GBM clonal diversity.[171] For example, hypermutation in GBM recurrence is only observed after TMZ treatment.[171,172] Recurrent tumors can exhibit both divergent evolution – in which the recurrent tumor is comprised of clones absent from the primary mass – and linear evolution, in which the predominant clones at recurrence existed in the original mass.[173] The predominating TCGA bulk expression subtype also commonly differs between primary and recurrent tumors,[174] with an observable predominance of the mesenchymal subtype at recurrence, suggesting that therapy induces genetic changes that converge on common pathways.

9.4. Stem-like cell enrichment

GBM stem-like cells are a slow-cycling population of GBM cells that represent a small percentage of the overall tumor mass but function as the cornerstone of tumorigenesis and therapeutic resistance in GBM.[175-178] GBMSCs are fundamentally defined by the capacity to self-renew and the ability to differentiate into a heterogeneous complement of progeny cells.[179] Thus, GBMSC clones occupy the multipotent apex of the GBM cellular hierarchy and exhibit substantial epigenetic and transcriptional plasticity that allows them to evade apoptosis.[180,181] Notably, GBMSCs can robustly activate DNA damage response pathways (178), autophagy,[182] and high expression of drug efflux transporters.[183]

It remains unclear whether GBMSCs arise from acquired mutations in normal neural stem cells (NSCs)[184] or develop through dedifferentiation of more terminally differentiated mutant glia,[170] but GBMSCs and NSCs share many biological features that underlie the clinical difficulties posed by GBMSCs. For example, GBMSCs reside in specific oncogenic niches that closely mirror neurogenic niches.[139] Interestingly, adult gliomas predominantly develop adjacent to or in direct contact with the lateral ventricular system, where the subventricular zone of adult neurogenesis also resides.[185] GBMSCs and NSCs express similar markers, such as CD133, Nestin, oligodendrocyte transcription factor 2 (OLIG2), and GFAP. They also rely on the activation of intersecting growth pathways such as Notch, epidermal growth factor, and fibroblast growth factor.[186] The counterbalancing pathways that restrain growth in NSCs are notably absent or dysregulated in GBMSCs.[186] Both GBMSCs and NSCs also form a three-dimensional neurosphere *in vitro* and give rise to fast-cycling and highly migratory progenitors.[139] GBMSCs also exhibit activity-dependent growth that includes significant downregulation of inhibitory γ-aminobutyric acid receptors and upregulation of glutamate signaling.[187,188] Understanding normal NSC function may highlight targetable dysfunction of similar pathways in GBMSCs.

10. Concluding remarks

The glioma research community has increasingly acknowledged that a lack of integration between cancer biology and neuroscience investigations has hindered progress in understanding glioma pathobiology. Correspondingly, the glioma research framework is steadily evolving toward a collective understanding that meaningful progress in treating diffuse glioma will require coordinated collaboration between cancer biologists and neuroscientists.[189] These emerging collaborative efforts aim to understand how the unique physiologic properties of the CNS fundamentally shape glioma development and aggression. Such studies now serve as the foundation for the burgeoning field of cancer neuroscience that has rapidly expanded beyond gliomas to additionally ignite diverse

investigations into the ways human cancers are shaped by the unique features of both central and peripheral nervous tissue.[190]

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Conflict of interest

The authors declare they have no competing interests.

Author contributions

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ACCSCIENCE PUBLISHING

Tumor Discovery

REVIEW ARTICLE

Honokiol in cancer: Roles in enhancing combination therapy efficacy and preventing post-transplant malignancies

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Abstract

Therapeutic resistance remains a significant challenge in cancer treatment, often resulting in relapse and poor outcomes. Conventional chemotherapies, such as cisplatin and paclitaxel, are frequently undermined by the development of chemoresistance and systemic toxicity. Targeted therapies, such as receptor tyrosine kinase (RTKs) inhibitors and monoclonal antibodies (mAbs), offer better specificity but face resistance over time. Combination therapies are being explored to improve efficacy and mitigate resistance. Honokiol, a biphenolic natural compound derived from *Magnolia* species, has emerged as a potential adjunct in combination therapies due to its anti-cancer, anti-inflammatory, and immunomodulatory properties. It enhances the efficacy of chemotherapies, such as cisplatin and paclitaxel, RTK inhibitors, such as cabozantinib and erlotinib, and mAbs, such as cetuximab. Notably, honokiol combined with mAbs has shown promise in pre-clinical studies by reactivating the immune system and reducing tumor growth in resistant models. In addition, honokiol aids in post-transplant cancer prevention by modulating immune responses, reducing tumor progression, and lowering the required dose of immunosuppressants, such as cyclosporine A and rapamycin. Pre-clinical studies in renal cell carcinoma (RCC), head and neck squamous cell carcinoma (HNSCC), and non-small cell lung cancer emphasize its potential to overcome resistance. Despite promising evidence, further clinical studies are needed to validate honokiol as a viable adjunct in combination therapies. While several reviews have focused on the effects of honokiol alone, there is a lack of comprehensive studies examining its potential in combination with other therapies. This review aims to fill this gap by offering critical insights into the role of honokiol as a candidate for combination therapy.

Keywords: Honokiol; Cancer; Combination therapy; Chemotherapy; Receptor tyrosine kinase inhibitors; Post-transplantation cancer

1. Introduction

Therapeutic resistance is a significant barrier to achieving durable responses in cancer treatment.[1] Despite considerable advances in the development of chemotherapies, targeted therapies, and monoclonal antibodies (mAbs), resistance, both intrinsic and acquired, continues to drive treatment failure, tumor progression, and poor patient prognosis.[2] Conventional chemotherapeutic agents, such as paclitaxel and doxorubicin, have long been the cornerstone of cancer management. However, their non-specific cytotoxicity often results in dose-limiting toxicities and the emergence of resistant tumor clones.[3,4]

The introduction of molecular targeted therapies, particularly receptor tyrosine kinase (RTKs) inhibitors, marked a pivotal advancement in precision oncology.[5] Drugs, such as cabozantinib, lapatinib, erlotinib, and osimertinib, selectively inhibit key oncogenic drivers in various malignancies. However, resistance to these agents often develops through secondary mutations, bypass signaling, and activation of compensatory pathways. Similarly, mAbs such as cetuximab have transformed the treatment landscape of many cancers. Nevertheless, immune escape mechanisms and tumor microenvironment factors frequently limit their long-term efficacy.

Combination therapies are increasingly recognized as a strategic approach to overcoming therapeutic resistance.[6,7] In this context, bioactive natural compounds have gained significant interest due to their multitargeted actions, favorable safety profiles, and the ability to synergize with standard therapies.[8-14] Honokiol, a biphenolic compound derived from the bark and leaves of the *Magnolia* species, has demonstrated a broad spectrum of pharmacological properties, including anti-cancer, anti-inflammatory, antioxidant, and neuroprotective effects (Figure 1).[15-18] Importantly, honokiol has shown the potential to resensitize resistant cancer cells to chemotherapeutic agents and targeted therapies, while enhancing the efficacy of mAbs.[19] Mechanistically, honokiol modulates several key oncogenic and survival pathways, including phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase, signal transducer and activator of transcription 3 (STAT3), and nuclear factor kappa B (NF-κB), and can reverse epithelial-mesenchymal transition, inhibit angiogenesis, and restore immune surveillance.[20]

In addition to its role in restricting cancer cell proliferation, honokiol has emerged as a promising candidate for preventing post-transplantation malignancies. Immunosuppressive agents, such as cyclosporin A and rapamycin, commonly used to prevent graft rejection,

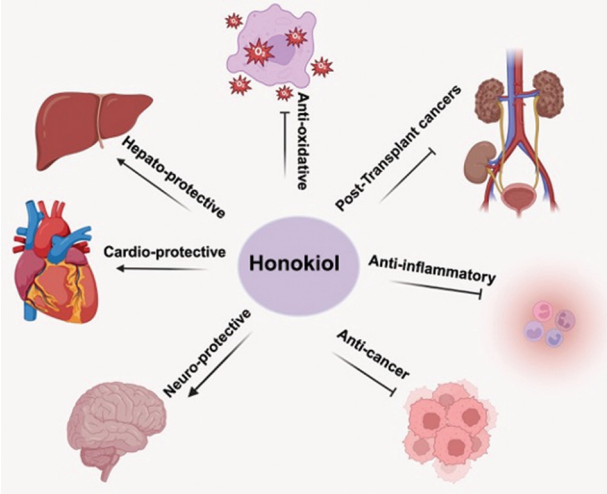

Figure 1. The figure illustrates the diverse biological activities of honokiol with broad therapeutic potential. Adapted and modified from our previously published article (*Phytochemistry Reviews,* 2025, Solanki *et al.*[18]), with copyright permission and license obtained from Springer Nature (Licence Number: 5986590534892).

can paradoxically promote tumorigenesis by suppressing immune surveillance and activating oncogenic pathways.[21,22] Honokiol, when combined with these immunosuppressants, has demonstrated efficacy in mitigating cancer-promoting signals while maintaining graft viability in pre-clinical models. This review aims to summarize the present pre-clinical evidence on honokiol, focusing on its role in combination therapies for cancer treatment, where its dual anti-inflammatory and anti-tumor effects may offer significant benefits.

2. Combination therapies with honokiol

Combination therapy involving honokiol has been extensively investigated in pre-clinical studies (Figure 2). Both *in vitro* and *in vivo* research have demonstrated that honokiol can enhance the efficacy of chemotherapy, radiation therapy, and targeted therapies across various cancers, including renal, oral, breast, lung, pancreatic, and colorectal cancer.[23-26] These studies suggest that honokiol could improve treatment outcomes when combined with conventional therapies.

2.1. Cisplatin and honokiol

Cisplatin, a widely used chemotherapy drug, is effective against cancers, such as ovarian, bladder, lung, and testicular cancer.[27] It damages DNA in cancer cells, preventing their division and proliferation. However, cisplatin's clinical use is limited by side effects such as nausea, kidney damage, hearing loss, and nerve toxicity. To overcome these limitations, cisplatin is often used in combination with other agents.

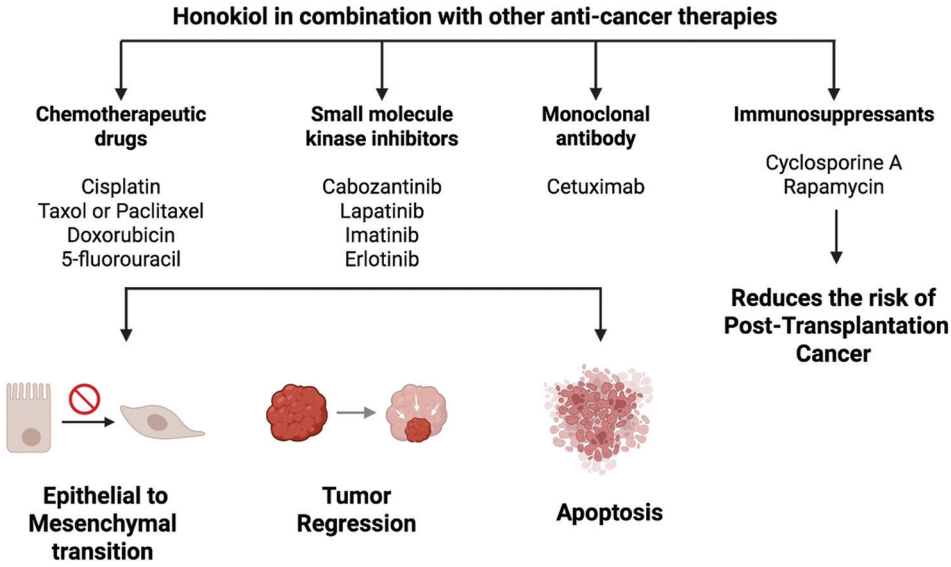

Figure 2. Summary of pre-clinical therapeutic combinations with honokiol. Image created in BioRender. Sabarwal, a. (2025) https://BioRender.com/i48w904.

Pharmacological studies indicate that combining cisplatin with honokiol significantly enhances its therapeutic efficacy while reducing side effects, particularly in colorectal and ovarian cancer.[28,29] For instance, honokiol has been shown to regulate interleukin 6 (IL-6)/STAT3 signaling pathway in oral carcinoma stem cells, sensitizing cancer cells to cisplatin.[30] In addition, when combined with curcumin, honokiol sensitizes multidrug-resistant lung cancer cells to cisplatin.[31]

A major limitation of cisplatin is nephrotoxicity. However, studies have demonstrated that honokiol pre-treatment reduced cisplatin-induced cytotoxicity by improving cell viability and reducing lactate dehydrogenase release. Honokiol also mitigates oxidative stress by reducing reactive oxygen species (ROS) and enhancing antioxidant enzyme activity in renal epithelial cells.[32] Honokiol has also been shown to protect against cisplatin-induced acute kidney injury in animal models by modulating mitochondrial fission and regulating key proteins such as dynamin-related protein 1 (DRP1)[33] and sirtuin 3.[34] Moreover, honokiol enhances therapeutic responses when combined with carboplatin and gemcitabine in docetaxel-resistant tumors.[19]

2.2. Paclitaxel (Taxol) and honokiol

Paclitaxel, a taxane chemotherapy agent, stabilizes microtubules and prevents cell division.[35] It is widely used to treat various cancers, including breast, ovarian, and lung cancers. The combination of paclitaxel and honokiol has been studied with promising results in several cancer models. A study by Wang *et al.*[36] demonstrated that the combination of honokiol and paclitaxel synergistically affected the multidrug-resistant human squamous KB cells *in vitro*. This combination also significantly inhibited tumor growth in a subcutaneous model, which was accompanied by a decrease in antigen Kiel 67 expression (a marker of cell proliferation) and an increase in terminal deoxynucleotidyl transferase dUTP nick end labeling-positive cells (indicative of apoptosis).[32] In lung cancer models, the combination of honokiol and paclitaxel-induced significant cell death in both sensitive (H1650, H1299) and resistant (H1650/PTX) cells through paraptosis, a form of programmed cell death involving vacuolation. This effect was observed in both *in vitro* and xenograft tumor models.[37]

Further studies have shown that dequalinium-modified paclitaxel combined with honokiol micelles exhibits promising therapeutic effects against non-small-cell lung cancer (NSCLC).[38]

The combination suppressed vasculogenic mimicry channels and tumor metastasis by activating apoptotic enzymes such as caspase-3 and caspase-9, and down-regulating key pathways, such as focal adhesion kinase, PI3K, matrix metallopeptidase (MMP)-2, and MMP-9. *In vivo* data revealed selective accumulation of these micelles at tumor sites, providing targeted antiproliferative effects.[38]

Wang *et al.*[38] also explored the use of pH-sensitive polymeric micelles to co-encapsulate paclitaxel and honokiol, achieving suppression of multidrug resistance and metastasis in breast cancer cells. The micelles reversed multidrug resistance by down-regulating P-glycoprotein expression and increasing plasma membrane fluidity.[39]

Similarly, Lu *et al.*[39] demonstrated that paclitaxel combined with honokiol nanosuspensions, encapsulated in thermosensitive hydrogels, allowed for sustained and targeted drug release at the tumor site.[40] Honokiol has also shown benefits as a complementary therapy in patients with paclitaxel-resistant tumors, particularly when administered intravenously.[19]

2.3. Doxorubicin and honokiol

Doxorubicin, a potent chemotherapeutic agent used for advanced-stage cancers, is known for its high toxicity, particularly cardiotoxicity.[41] To mitigate these effects, doxorubicin is often administered in combination with other agents to enhance efficacy while reducing toxicity.[42,43] Studies have investigated the combination of doxorubicin and honokiol, which has shown promise in complementing doxorubicin's anticancer effects while mitigating cardiotoxicity. Honokiol has been shown to reverse doxorubicin resistance in human breast cancer cells by targeting a molecular pathway involving microRNA-188-5p, *FBXW7*, and *c-Myc*. Honokiol increases the expression of microRNA-188-5p, which upregulates *FBXW7*, a tumor suppressor gene that downregulates *c-Myc*, effectively reversing drug resistance and inhibiting tumor growth.[44] Moreover, honokiol enhances doxorubicin's efficacy by regulating mucin 1 and multidrug resistance protein 1, further improving its therapeutic effects and reducing the likelihood of resistance.[45] Importantly, honokiol's cardioprotective properties provide a significant advantage, offering a safer combination therapy for patients receiving doxorubicin.

2.4. 5-Fluorouracil (5-FU) and honokiol

5-FU, a pyrimidine analog, is a widely used chemotherapeutic agent that inhibits nucleic acid synthesis, thereby suppressing cancer cell growth and proliferation. However, its clinical utility is often limited by toxicity and resistance.[46]

Several studies have explored the combination of 5-FU with honokiol, demonstrating enhanced efficacy and reduced side effects. Ji *et al.*[46] investigated this combination in oral squamous cell carcinoma cells and *in vivo* models. Their findings revealed that the combination induced significantly higher levels of apoptosis and suppressed tumor growth more effectively than either agent alone.[47]

Similarly, honokiol induced apoptosis in human urothelial cell carcinoma cells and caused G0/G1 cell cycle arrest. When combined with 5-FU, honokiol exhibited a synergistic effect, further enhancing the therapeutic response.[48] Swidan *et al.*[48] reported that combining 5-FU and nanoparticulated honokiol significantly reduced tongue carcinoma induced by 4-nitroquinoline oxide in Wistar albino rats. Notably, this combination therapy also decreased systemic toxicity compared to either treatment alone.[49] These findings suggest that honokiol can potentiate the anti-tumor effects of 5-FU while mitigating its adverse effects, making it a potential adjunct in cancer therapy.

2.5. Metformin and honokiol

Metformin, an established anti-diabetic medication, has gained attention for its potential anticancer effects. By lowering systemic glucose levels, metformin limits the energy supply available to cancer cells, thereby inhibiting their growth and proliferation. Studies have shown that combining metformin with honokiol yields promising synergistic effects. In hormone-resistant breast cancer cells (MCF7/HT), the combination of honokiol and metformin effectively inhibited cell proliferation and induced apoptosis. This suggests that the dual treatment may overcome resistance mechanisms common in hormone-independent breast cancers, enhancing therapeutic efficacy.[50] These findings highlight the potential of honokiol and metformin as a combination strategy to exploit metabolic vulnerabilities in cancer cells. Further research could establish this regimen as a viable therapeutic approach for hormone-resistant cancers.

2.6. Bleomycin and honokiol

Bleomycin is an important chemotherapeutic agent used in the treatment of Hodgkin lymphoma and testicular germ-cell tumors, two of the most curable cancers. However, its clinical application is frequently limited by serious pulmonary side effects, including hypersensitivity pneumonitis, bronchiolitis obliterans organizing pneumonia, acute interstitial pneumonia, and progressive pulmonary fibrosis.[51] Combining honokiol and bleomycin has enhanced anticancer efficacy while potentially reducing toxicity. In breast cancer (MCF7), pancreatic cancer (PANC-1), and melanoma (UACC903) cell lines, honokiol reduced the effective concentration of bleomycin by tenfold. This enhanced potency is attributed to honokiol's ability to inhibit the repair of bleomycin-induced single- and double-strand DNA damage, thereby promoting cancer cell death. By enabling lower therapeutic doses of bleomycin, this combination may help minimize pulmonary side effects while maintaining or improving anticancer activity.[52] These findings suggest that honokiol could serve as an effective adjuvant to bleomycin-based chemotherapy.

3. Monoclonal antibody and honokiol combination

Monoclonal antibodies have revolutionized cancer therapy by targeting tumor-associated antigens, improving

treatment precision, and minimizing damage to normal tissues. However, drug resistance and limited efficacy in some patient populations are challenging.

Honokiol, combined with mAbs, has shown the potential to overcome these limitations by enhancing therapeutic responses and mitigating resistance mechanisms. For example, cetuximab is an anti-epidermal growth factor receptor (EGFR) monoclonal antibody approved for treating head and neck squamous cell carcinoma (HNSCC) and metastatic colorectal cancer. Despite its efficacy, resistance to cetuximab frequently develops. Pearson *et al.*[52] demonstrated that combining honokiol with cetuximab produced significant antiproliferative effects in cetuximab-resistant cancer cells.[53] The combination downregulated the human epidermal growth factor receptor (HER) family members and inhibited associated signaling pathways, including MAPK and AKT. Furthermore, honokiol reduced the phosphorylation of DRP1 and levels of ROS, indicating altered mitochondrial function. The combination therapy was also validated in cetuximab-resistant HNSCC patient-derived xenograft models, where it led to a notable delay in tumor growth and decreased activation of MAPK, AKT, and DRP1 signaling, consistent with *in vitro* findings.

These results highlight honokiol's potential to overcome resistance to cetuximab and enhance the efficacy of mAb-based therapies. In addition, honokiol's ability to modulate key signaling pathways and counteract resistance mechanisms supports its use as a promising adjunct in combination therapies involving mAbs and other targeted agents.

4. Honokiol in combination with small-molecule inhibitors (SMIs)

SMIs play a central role in modern oncology, offering targeted inhibition of signaling proteins and pathways critical to tumor growth and survival.[54] They effectively block RTKs, such as EGFR, mesenchymal-epithelial transition factor (MET), and vascular endothelial growth factor receptors (VEGFR), intracellular signaling mediators, such as MAPK kinase and PI3K, and apoptotic regulators, including B-cell lymphoma 2.[55] However, challenges such as acquired resistance and toxicity limit their long-term success. Honokiol has emerged as a promising agent in combination with SMIs, enhancing their anti-tumor efficacy while helping to overcome resistance and reduce side effects.[24,56-58]

4.1. Cabozantinib (XL-184) and honokiol

Cabozantinib is a multi-kinase inhibitor targeting c-MET, VEGFRs, and other RTKs and has demonstrated significant efficacy in cancers such as renal cell carcinoma (RCC). Despite its clinical success, tumor resistance often develops, limiting its long-term benefit.

Recent studies from our laboratory investigated the synergistic effects of cabozantinib and honokiol in RCC models. The studies focused on the role of the c-MET RTK in cancer progression and resistance. Hyperactivation of c-MET promotes cancer cell survival by activating pathways that help them withstand oxidative stress, contributing to drug resistance.[26,57]

Mechanistic investigations identified proteins such as Rubicon and p62, which regulate autophagy and oxidative stress, along with the transcription factor nuclear factor erythroid 2-related factor 2, as key players in resistance. The combination of cabozantinib and honokiol significantly inhibited RCC cell proliferation *in vitro* and reduced tumor growth *in vivo* in xenograft models. Moreover, this combination therapy decreased the expression of Rubicon, p62, and heme oxygenase-1, reducing tumor vascular density.[57]

These findings highlight honokiol's potential to enhance cabozantinib's anti-tumor efficacy and offer a promising strategy to overcome resistance in RCC treatment.

4.2. Lapatinib and honokiol

Lapatinib is a dual tyrosine kinase inhibitor targeting EGFR and HER2, primarily used to treat HER2-positive breast cancer. Despite its efficacy, resistance, and toxicity remain concerns in clinical practice.

Honokiol has demonstrated broad anticancer activity in various breast cancer cell lines, including estrogen receptor-positive, estrogen receptor-negative, and drug-resistant lines (e.g., adriamycin- and tamoxifen-resistant cells).[24] It induces G1-phase cell cycle arrest and caspase-dependent apoptosis in a time- and dose-dependent manner. Notably, HER2 knockdown increases cellular sensitivity to honokiol-induced apoptosis in HER2-overexpressing cells.

The combination of honokiol and lapatinib significantly amplifies anti-tumor effects in HER2-overexpressing breast cancer models. Mechanistically, honokiol downregulates AKT phosphorylation and upregulates *PTEN* expression, resulting in suppression of the PI3K/AKT/mammalian target of rapamycin (mTOR) pathway, an essential driver of cancer cell survival and proliferation.[24] These findings support the potential of combining honokiol with lapatinib as a novel strategy for HER2-positive breast cancer.

4.3. Imatinib and honokiol

Imatinib is a well-established targeted therapy for chronic myeloid leukemia and gastrointestinal stromal tumors. Despite its success, resistance and incomplete responses necessitate a combination approach.

Wang *et al.*[58] demonstrated that honokiol induces two distinct forms of cell death in leukemia cells: Paraptosis at lower concentrations (characterized by cytoplasmic vacuolization and endoplasmic reticulum swelling) and apoptosis at higher concentrations. These processes may occur sequentially or in parallel, depending on honokiol dosage.

In addition, honokiol disrupts leukemia cell adhesion to the extracellular matrix in a concentration-dependent manner, potentially reducing metastatic potential. Sequential treatment administering honokiol before imatinib exhibited synergistic effects, enhancing imatinib's therapeutic efficacy in K562 leukemia cells.[59]

These findings suggest that honokiol's dual-mode induction of cell death, combining apoptotic and non-apoptotic mechanisms, may offer a novel approach for improving imatinib responses in leukemia treatment.

4.4. Erlotinib and honokiol

Erlotinib, an EGFR inhibitor, is widely used to treat HNSCC and NSCLC. However, its long-term efficacy is often limited by the development of resistance, necessitating alternative therapeutic strategies. Leeman-Neill *et al.*[59] investigated honokiol as a potential therapeutic agent for HNSCC, focusing on its ability to target EGFR signaling. Honokiol inhibited tumor cell proliferation (half maximal effective concentration: 3.3 – 7.4 μM), induced apoptosis, and suppressed key EGFR downstream signaling pathways, including MAPK, AKT, and STAT3. In addition, honokiol enhanced the efficacy of erlotinib, leading to significant tumor growth inhibition *in vivo*.[56]

Another study further demonstrated honokiol's potential in inhibiting lung cancer cell growth, driven by EGFR deregulation. Honokiol at concentrations 2.5 – 7.5 μM suppressed cell proliferation by up to 93% and induced apoptosis in 61% of EGFR-overexpressing bronchial cells. It also downregulated phosphorylated EGFR, AKT, STAT3, and cell cycle-related proteins within 6 – 12 h of treatment. Interestingly, although honokiol exhibited weaker direct EGFR tyrosine kinase binding compared to erlotinib, its overall antiproliferative and pro-apoptotic effects were stronger, suggesting inhibition of additional critical survival pathways. Furthermore, honokiol sensitized erlotinib-resistant cells to erlotinib and significantly reduced lung tumor size and multiplicity by 49% in mouse models. These findings suggest honokiol's potential as both a monotherapy and an adjuvant strategy for overcoming erlotinib resistance in EGFR-driven cancers.[60]

4.5. Osimertinib and honokiol

Osimertinib is a third-generation, Food and Drug Administration-approved EGFR inhibitor that targets *EGFR*-T790M mutations in NSCLC. Despite its clinical success, resistance develops, often due to additional mutations such as C797S, posing a major therapeutic challenge. Honokiol has shown promise in overcoming acquired resistance to osimertinib. In pre-clinical studies, the combination of honokiol and osimertinib synergistically reduced cell viability and colony formation in osimertinib-resistant NSCLC cell lines. This combination also significantly enhanced apoptosis compared to either agent alone.

In mouse xenograft models harboring *EGFR* 19del, T790M, and C797S triple mutations, co-treatment with honokiol and osimertinib effectively suppressed tumor progression. Importantly, the combination was well-tolerated, with no significant toxicity observed in the treated mice. Mechanistic analyses revealed that the combination therapy inhibited phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and promoted degradation of anti-apoptotic protein myeloid cell leukemia-1, leading to enhanced induction of apoptosis.[61] These findings support further clinical evaluation of honokiol and its derivatives as adjuvants to overcome osimertinib resistance in *EGFR*-mutant NSCLC.

5. Honokiol as an anti-inflammatory agent

Inflammation plays a dual role in disease development, particularly in cancer. Chronic inflammation can be pro-tumorigenic due to the sustained presence of pro-inflammatory cytokines, which promote tumor cell proliferation, survival, angiogenesis, and metastasis. Conversely, acute inflammation can exert anti-tumorigenic effects by enhancing immune surveillance, promoting tumor-associated antigen presentation, and influencing immune cell polarization. Honokiol has been extensively studied for its potent anti-inflammatory properties, contributing to its anti-cancer effects. It inhibits the production of key pro-inflammatory cytokines, including tumor necrosis factor-alpha, IL-1 beta, and IL-6, across various cell types.[62-64] In addition, honokiol attenuates the activation of critical inflammatory signaling pathways, particularly NF-κB, a key regulator of inflammation. By inhibiting protein kinase C and MAPKs, honokiol disrupts phosphorylation events essential for inflammatory signaling cascades.[65,66] These properties make honokiol a compelling candidate for modulating tumor-associated inflammation and enhancing the efficacy of anti-cancer therapies.

6. Post-transplantation cancer and the role of honokiol in its prevention

Post-transplantation cancers are malignancies that develop in organ or hematopoietic stem cell transplant recipients, primarily due to prolonged immunosuppressive therapy

aimed at preventing graft rejection. These therapies, while essential for transplant success, compromise immune surveillance and increase susceptibility to oncogenic viruses and malignancies such as skin cancers, Kaposi sarcoma, and lymphomas, including post-transplant lymphoproliferative disorders[67] Oncogenic viruses, such as Epstein-Barr virus, human papillomavirus, and human herpesvirus 8 are frequently implicated in these malignancies.[68,69] Other factors, such as recipient age at the time of transplantation, gender, and genetic pre-disposition, further modulate cancer risk.[70,71] The present management strategies emphasize regular cancer screening, modulation of immunosuppressive regimens, targeted therapies, and vaccinations against oncogenic viruses.[72,73]

Honokiol has demonstrated potential as an adjuvant therapy to mitigate the increased cancer risk associated with post-transplant immunosuppression. Its anti-inflammatory, antiproliferative, and immunomodulatory properties make it an attractive candidate for integration into post-transplant cancer prevention strategies.

6.1. Cyclosporine A and honokiol

Cyclosporine A (CsA) is a calcineurin inhibitor widely used to prevent transplant rejection. It blocks the translocation of the nuclear factor of activated T cells to the nucleus, suppressing T cell activation and immune responses. However, CsA also promotes tumor progression by activating oncogenic pathways such as Ras-Raf-ERK

Table 1. Combination treatments with honokiol

Drug/therapeutic name	Cancer type/models	Key findings	References
Chemotherapeutic drugs			
Cisplatin	Colorectal cancer, ovarian cancer, oral cancer, lung cancer, and renal cell carcinoma	Reduce toxicity, re-sensitization, interleukin-6/STAT3 regulation, dynamin-related protein 1 regulation, and reactive oxygen species and anti-oxidative enzyme regulation	19,28-34
Paclitaxel (Taxol)	Human squamous KB cells, lung cancer, and breast cancer	Inhibit cell proliferation and tumor growth, induce paraptosis, downregulation of focal adhesion kinase, PI3K, MMP-2, and MMP-9	19,37-40
Doxorubicin	Breast cancer and cardiomyopathy	Inhibit growth and proliferation by regulating microRNA-188-5p, *FBXW7*, and c-*Myc*, regulation of mucin 1 and multidrug resistance protein 1, and cardioprotective properties	42-45
5-fluorouracil	Oral cancer, urothelial cell carcinoma, and tongue cancer	High apoptosis, suppresses tumor growth, cell cycle arrest, and decreased systemic toxicity	47-49
Metformin	Breast cancer	Induce apoptosis and inhibit cell growth	50
Bleomycin	Breast cancer, pancreatic cancer, and melanoma	Reduce pulmonary toxicity and inhibit DNA repair	52
Monoclonal antibodies			
Cetuximab	Cetuximab-resistant cancer cells	Resensitization, regulate HER, MAPK, AKT, and dynamin-related protein 1 pathways	53
Small-molecule inhibitors			
Cabozantinib	Renal cell carcinoma	Induce reactive oxygen species-mediated apoptosis and autophagy, inhibit Rubicon, p62, and HO-1	57
Lapatinib	Breast cancer	Cell cycle arrest induces apoptosis, suppresses PI3K/AKT/mTOR pathway	24
Imatinib	Leukemia	Inhibit cell adhesion to the extracellular matrix and induce paraptosis	59
Erlotinib	Head and neck squamous cell carcinoma and lung cancer	Induce apoptosis, inhibit EGFR signaling pathways, including MAPK, AKT, and STAT3	56,60
Osimertinib	Non-small cell lung cancer	Inhibit cell proliferation and induce apoptosis, suppress tumor growth even with 19del, T790M, and C797S triple mutations, inhibit p-ERK1/2, and promote myeloid cell leukemia-1 degradation	61
Immunosuppressive drugs in transplantation			
Cyclosporine A	Renal cell carcinoma	Inhibit cyclosporine A-induced Ras-Raf-ERK and VEGF pathways	74
Rapamycin	Renal cell carcinoma	Inhibit cell proliferation and growth, inhibit Rubicon, programmed death-ligand 1, c-mesenchymal-epithelial transition factor, and AXL, and downregulate HO-1	26,77

Abbreviations: AKT: Protein kinase B; EGFR: Epidermal growth factor receptor; ERK: Extracellular signal-regulated kinase; HER: Human epidermal growth factor receptor; HO-1: Heme oxygenase-1; MAPK: Mitogen-activated protein kinase; MMP: Matrix metallopeptidase; mTOR: Mammalian target of rapamycin; PI3K: Phosphoinositide 3-kinase; STAT3: Signal transducer and activator of transcription 3; VEGF: Vascular endothelial growth factor.

and vascular endothelial growth factor signaling. Our research demonstrated that honokiol, administered alone or in combination with CsA, effectively inhibits these cancer-promoting pathways in RCC models.[74] Moreover, honokiol's anti-inflammatory effects may allow for dose reduction of CsA without compromising graft survival, potentially reducing its oncogenic side effects.

6.2. Rapamycin and honokiol

Rapamycin (sirolimus), an mTOR inhibitor, is frequently employed to prevent organ rejection, particularly in renal transplantation. While rapamycin possesses inherent anti-tumor activity, prolonged treatment can activate compensatory survival pathways. Specifically, sustained rapamycin exposure relieves the negative feedback loop on AKT through inhibition of S6-kinase, potentially promoting tumor growth through PI3K-mTOR signaling.[75,76]

Sabarwal *et al.*[26] explored the therapeutic potential of combining honokiol with rapamycin in post-transplantation cancer models. This combination effectively inhibited the c-MET-driven proliferation of renal cancer cells. c-MET is a RTK commonly overexpressed in RCC and linked to tumor growth and metastasis. In addition, the combination downregulated programmed death-ligand 1, a key immune checkpoint molecule that facilitates tumor immune evasion.[26]

In a murine model of post-transplant renal cancer, the honokiol and rapamycin combination prolonged allograft survival and significantly inhibited tumor growth.[77] Mechanistically, this therapy modulated the expression of tumor-promoting regulators such as Carabin and Rubicon, induced autophagic and apoptotic cell death, and reduced the expression of the RTK AXL, reported to be overexpressed in various cancer types.[78] Notably, the combination also suppressed the expression of heme oxygenase-1, a cytoprotective enzyme implicated in therapeutic resistance. These findings highlight honokiol's potential as a novel adjunct therapy to mitigate post-transplant cancer risk while preserving graft survival.

7. Conclusion

Therapeutic resistance remains one of the most significant obstacles to effective cancer treatment, contributing to disease progression and treatment failure. In response, numerous therapeutic strategies have been developed to overcome this challenge. These include novel targeted therapies such as SMIs of RTKs, immune checkpoint inhibitors, and mAbs designed to specifically target resistant cancer subtypes. While these agents often elicit promising initial responses, they frequently lead to the emergence of more aggressive and therapy-resistant tumor clones.[79] Acquired resistance is primarily driven by the complex and adaptive nature of tumor architecture. Tumor cells dynamically remodel their microenvironment through physical and biochemical mechanisms, promoting immune evasion, migration, invasion, and resistance to apoptosis.[80] These adaptive changes create barriers to effective drug delivery and foster the survival of drug-resistant cancer cell populations. Combination therapies (Table 1) have emerged as a more effective strategy than single-agent treatments, as they simultaneously target multiple oncogenic pathways and enhance tumor cell eradication. Several combination regimens have already gained approval and are in clinical use, although further improvements in efficacy, safety, and tolerability are still needed.[81-83]

In this context, drug repurposing has gained traction as a viable strategy to reduce drug development costs and accelerate the translation of therapies into clinical practice. Natural compounds, including plant-derived bioactive molecules, have been extensively studied for their anticancer potential. Honokiol, in particular, has demonstrated potent anticancer activity across various malignancies, with additional preventive benefits.[84] Notably, honokiol has shown the ability to sensitize therapy-resistant cancer cells when used in combination with other conventional and targeted treatments.[29,36,38,42,49,53,59,60] Pre-clinical studies from our laboratory have further confirmed the therapeutic efficacy of honokiol in both cancer and post-transplantation settings. However, to fully elucidate its clinical potential, more in-depth investigations are warranted, including comprehensive pre-clinical studies to fully evaluate the potential of honokiol as a treatment option.

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Conflict of interest

The authors declare they have no competing interests.

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Availability of data

Data sharing does not apply to this article as no new data were created or analyzed in this study.

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ACCSCIENCE PUBLISHING

Tumor Discovery

ORIGINAL RESEARCH ARTICLE

Development and validation of a comprehensive tumor treating fields system for glioblastoma therapy: From prototype design to preclinical evaluation

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Abstract

Glioblastoma multiforme (GBM) is an aggressive and lethal brain tumor with limited treatment options and poor prognosis. Standard therapies such as surgery, radiation, and chemotherapy provide modest survival benefits but are often ineffective against tumor recurrence. Tumor treating fields (TTF) therapy has emerged as a promising non-invasive treatment modality that uses alternating electric fields to disrupt cancer cell division and inhibit tumor growth. However, the optimization and practical implementation of TTF systems remain challenging due to limitations in field penetration, electrode design, and treatment efficacy. In this study, we designed and developed a novel TTF prototype system to enhance electric field transmission and optimize therapeutic efficiency. The system incorporates high-dielectric ceramic electrodes made of barium titanate zirconate, allowing for superior field penetration. We evaluated the system through a series of *in vitro* and *in vivo* experiments. *In vitro*, GBM cells exposed to the TTF system exhibited significant reductions in proliferation, with higher field intensities yielding greater inhibition. *In vivo*, using a rat GBM model, we observed marked tumor suppression, as validated by bioluminescence imaging and magnetic resonance imaging. Survival analysis further demonstrated prolonged lifespan in TTF-treated rats compared to controls. Our findings highlight the potential of this novel TTF system to improve GBM treatment outcomes. This study provides a comprehensive framework for future advancements in TTF therapy, paving the way for clinical translation and further integration with conventional and emerging cancer therapies.

Keywords: Glioblastoma multiforme; Tumor therapy; Tumor treating fields; Electric field therapy; System design

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1. Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor in adults, representing approximately 15% of all brain tumors.[1-3] Despite

aggressive treatment approaches, including surgery, radiation, and chemotherapy, the prognosis for GBM patients remains dismal, with a median survival time of only 12 – 15 months.[4] The challenges in treating GBM lie in its highly invasive nature, rapid proliferation, and resistance to standard therapies. Current therapies are often limited by the blood-brain barrier, which restricts the delivery of chemotherapeutic agents, as well as the tumor's heterogeneity, which leads to treatment failure and recurrence.[5]

The standard treatment regimen for GBM, known as the Stupp protocol, includes maximal safe surgical resection followed by radiotherapy and concurrent temozolomide chemotherapy.[6,7] Although this approach slightly extends survival,[8] the 5-year survival rate of patients typically remains below 10%, underscoring the urgent need for novel therapeutic strategies. Moreover, extensive infiltration of GBM into healthy brain tissue renders complete surgical removal nearly impossible, necessitating adjuvant therapies capable of targeting residual tumor cells without causing significant toxicity to the surrounding brain structures.[9]

Tumor treating fields (TTF) have emerged as a promising non-invasive therapeutic modality for GBM, offering an alternative mechanism to target tumor growth.[10-12] TTF utilizes low-intensity, alternating electric fields at intermediate frequencies (100 – 300 kHz) to disrupt the mitotic process of rapidly dividing tumor cells.[13] These fields interfere with microtubule polymerization, alignment of chromosomes, and other essential mitotic functions, leading to apoptosis.[14,15] The U.S. Food and Drug Administration (FDA) has approved the use of TTF therapy through devices like the Optune system, further validating its potential in GBM treatment. Clinical trials have demonstrated the ability of TTF therapy to prolong overall survival and progression-free survival in GBM patients when combined with standard chemoradiation.[16-18] However, the full potential of TTF has yet to be realized, as current systems are limited in their flexibility to adjust field parameters, and few studies have explored the detailed engineering behind the design and optimization of TTF systems.

Despite its promising efficacy, TTF therapy is constrained by several limitations, including patient adherence to continuous treatment (at least 18 h/day),[19] variability in treatment response due to anatomical and tumor heterogeneity,[19] and potential skin irritation from electrode placement.[20] In addition, the precise biophysical mechanisms underlying TTF therapy remain an active area of research, with emerging studies investigating its broader impact on cell signaling pathways, immune modulation, and the tumor microenvironment.[21-26] Understanding these mechanisms is crucial for refining TTF parameters to enhance its therapeutic effectiveness and integrate it with other emerging treatments, such as immunotherapy and targeted drug delivery systems.

To address these limitations, our study presents the design, development, and preclinical evaluation of a novel TTF system, focusing on key components such as electrical signal regulation, transmission, and corresponding therapeutic effects. We designed the system to generate highly controlled electric fields and incorporated it with high-dielectric ceramic electrodes composed of barium titanate zirconate,[27-29] which have superior electric field transmission properties compared to conventional electrodes. By evaluating the performance of this system through *in vitro* glioblastoma cell experiments and *in vivo* rat models, we demonstrate its efficacy in inhibiting tumor growth. Furthermore, we provide a comprehensive guide to the technical design of the system to further democratize TTF research more accessible with the hope to deepen innovation in the field.

This study contributes valuable insights into the optimization of TTF therapy for clinical use, particularly in enhancing the effectiveness of electric field-based cancer treatments. In addition, we explore how electric field intensity, frequency, and electrode design impact treatment outcomes, providing data that could inform future advancements in personalized TTF therapy. Our approach integrates interdisciplinary expertise in engineering, materials science, and oncology, positioning this study as a crucial step toward improving the clinical application of TTF therapy. By systematically analyzing treatment parameters and their biological effects, we aim to bridge the gap between experimental and clinical applications, ultimately refining TTF therapy as a viable component of multimodal GBM treatment strategies.

Beyond the immediate implications for GBM treatment, the principles underlying TTF therapy may hold broader applications in other malignancies, including lung,[30,31] pancreatic,[32,33] and ovarian[34,35] cancers. Future research should explore the feasibility of expanding TTF technology to different cancer types, optimizing electrode configurations, and integrating real-time monitoring systems to enhance treatment precision. By advancing TTF system design and understanding its mechanistic effects, we hope to contribute to the development of more effective, personalized, and patient-friendly cancer therapies.

2. Materials and methods

2.1. Materials and instruments

The reagents used in the cell experiments comprised glioblastoma cells (Cyagen Biosciences), Dulbecco's

Modified Eagle medium (Procell Life Science and Technology Co., Ltd.), penicillin-streptomycin (Procell Life Science and Technology Co., Ltd.), fetal bovine serum (ExCell Bio). The reagents used in the rat experiments comprised C6-LUC glioma cells (Cyagen Biosciences), isoflurane (RWD Life Science Co., Ltd.), D-Luciferin Potassium Salt (Rhone Reagents), and Sprague-Dawley (SD) rats sourced from Hangzhou Qizhen Experimental Animal Technology Co., Ltd. All rats were male, aged between 6 and 8 weeks. Ceramic electrodes were acquired from the Flexible Bioelectronics Division, Institute of Flexible Electronics Technology of THU.

The instruments used for the electrode test comprised Bruker D8 Discover X-ray diffractometer (XRD), Zeiss Sigma 300 scanning electron microscope, and Agilent 4294A precision impedance analyzer. The instruments used for the rat experiments comprised stereotaxic apparatus (Blue Star B/S, Anhui Zhenghua Biological Instrument Co., Ltd.), multimode small animal *in vivo* imaging system (AniView 600, Guangzhou Boluoteng Biotechnology Co., Ltd.), small animal anesthesia machine (Model H1670401-200L, RWD Life Science Co., Ltd.), small animal magnetic resonance imaging (MRI) system (7.0T MRI, Bruker Biospin GmbH PharmaScan7016), and constant-temperature drying oven (Model DHP-9082, Ningguo Shaying Scientific Instrument Co., Ltd.).

2.2. Cell experiments

Four round glass slides were placed in the TTF cell experimental device. Three milliliters of cell suspension (density 2×10^5 cells/mL) was applied evenly on a slide, and incubated at 37°C. The TTF-treated group was subjected to the alternating electric field with specific parameters (field strength and frequency). Control cells received no electric field stimulation but were otherwise cultured under identical conditions. The slides were taken out at specified times, and the cells were digested and counted. Cell morphology and proliferation rates were recorded at each time point, and changes in cell density were analyzed to assess the effect of TTF on cell growth.

2.3. Rat tumor model

Rats were acclimatized in a specific pathogen-free (SPF)-grade environment at a controlled temperature of 20 – 26°C and humidity of 40 – 70%, with a 12-h light-dark cycle. The rats were provided sterilized corn cob bedding, along with free access to food and water for 7 days. Following acclimation, the rats were divided into two groups: the control group and the TTF treatment group, with three rats in each group.

The glioblastoma model[36,37] was established by stereotaxic injection of C6-LUC cells. The rats were anesthetized using isoflurane and secured in a stereotaxic frame. The C6-LUC cells, harvested during their exponential growth phase, were prepared at a concentration of 5×10^5 cells in 5 μL PBS. The cells were injected into the right striatum at coordinates 1 mm posterior to bregma, 3 mm lateral to the midline, and 4.5 mm deep from the skull surface. After injection, the skull was sealed with bone wax, and the incision was sutured and disinfected. The rats were then returned to the SPF environment for recovery.

2.4. TTF treatment

Three days post-injection, TTF treatment was administered. Electrode patches were affixed to the shaved scalp of anesthetized rats, positioned orthogonally to create intersecting electric fields. The TTF system was activated for >20 h daily, with continuous treatment until day 20. The electric field parameters were set to 2 V/cm at 200 kHz, matching the *in vitro* settings. Every 4 days, the electrodes were removed, the skin was disinfected, and fresh electrodes were attached.

Rats were monitored daily for general health, and the skin reactions at the electrode sites were recorded. Bioluminescent imaging was conducted on days 10, 12, 14, 16, and 18 using an *in vivo* imaging system following intraperitoneal injection of D-luciferin. MRI scans were performed on day 19 using a T2-TurboRARE sequence to confirm tumor size. On day 19, blood samples were collected for complete blood count and biochemical analysis.

3. Results

A block diagram is a graphical representation of a system's architecture, showing key components and their interactions. It provides a high-level overview of the system's functionality, which is crucial for understanding how different subsystems work together. In this study, the overall design block diagram of the TTF system is shown in Figure 1A, including a Signal Generator, Signal Amplifier, and Control Module. The Signal Generator produces a 200 kHz sine wave, amplified by the Signal Amplifier, and modulated through a Microcontroller Unit-controlled Control Module to adjust signal intensity and frequency for each of the 36 output channels. This modular design allows independent control over each electrode's output, enabling precise application of electric fields. In the cell culture experiments, four electrodes are arranged orthogonally, providing alternating electric fields every second, as depicted in the inset of Figure 1A.

A printed circuit board (PCB) is the backbone of modern electronic systems, providing mechanical support and electrical connections for components. It

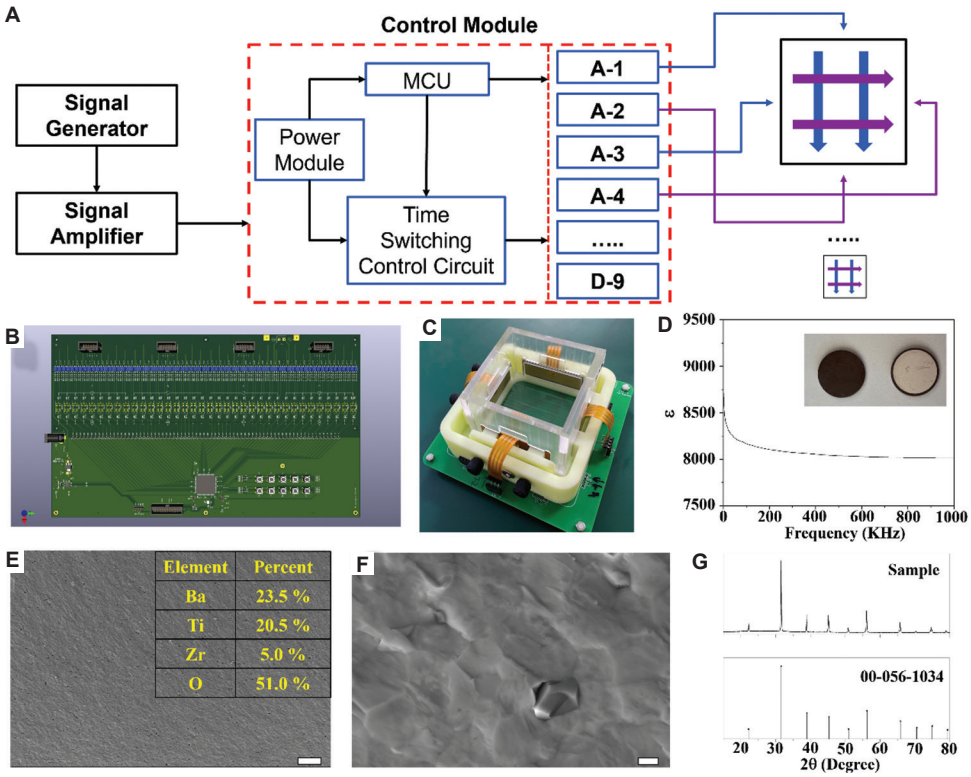


Figure 1. Overview of TTF system. (A) The overall design block diagram of the TTF system. (B) The PCB layout of the Control Module. (C) An actual photograph of the TTF system used in the cell experiments. (D) The measurements of the ceramic electrode across different frequencies; the inset shows the front and back of a ceramic electrode, with the back silver plated. (E and F) SEM images and EDS analysis showing the cross-sectional morphology of the ceramic electrode. Scale bars: 100 μm (e); 2 μm (f). (G) The XRD pattern of the ceramic electrode.
Abbreviations: EDS: Energy dispersive spectrometry; PCB: Printed circuit board; SEM: Scanning electron microscopy; TTF: Tumor treating fields; XRD: X-ray diffractometry.

consists of multiple layers of insulating material and conductive traces that facilitate signal transmission. In this study, the PCB layout is shown to illustrate how the Control Module distributes electric signals precisely to each electrode. The Control Module's PCB layout, shown in Figure 1B, highlights the intricate design needed to manage the precise delivery of the electric fields to each electrode. Figure 1C presents the prototype used in the cell experiments, demonstrating the physical implementation of the system with orthogonally positioned ceramic electrodes. A key feature of the system is the high-dielectric ceramic electrodes, which have a relative dielectric constant exceeding 8000 at 200 kHz, significantly higher than the reported electrodes[6] (Figure 1D). The superior dielectric properties ensure effective electric potential transmission during treatment. The electrode's cross-sectional morphology, analyzed by the scanning electron microscope, reveals a uniform structure, and the energy dispersive spectrometer confirms the primary composition as barium titanate zirconate (Figure 1E and 1F). The XRD analysis (Figure 1G) further validates the crystalline structure of the ceramic electrodes, matching the standard PDF card 00-056-1034, confirming the suitability for stable and efficient electric field generation. The combination of these images confirms the successful design and fabrication of the TTF system, ensuring effective electric field delivery during therapy.

In the cell culture experiments, a well-defined electrode arrangement was used to apply alternating electric fields to glioblastoma cells, allowing for a controlled investigation of TTF effects on cell proliferation. The schematic diagram of the experimental setup helps illustrate how electric fields interact with cells. The glioblastoma cells (U251) were exposed to varying electric field intensities using the TTF system. The experimental setup is illustrated in Figure 2A, where four circular cell culture slides were placed under orthogonally arranged electrodes to apply the electric fields in alternating directions. Finite element analysis is a numerical method used to simulate and analyze physical phenomena such as electric fields, thermal distribution, and mechanical stress. In Figure 2B, the electric field distribution across the culture area was simulated using the finite element analysis software (COMSOL Multiphysics),

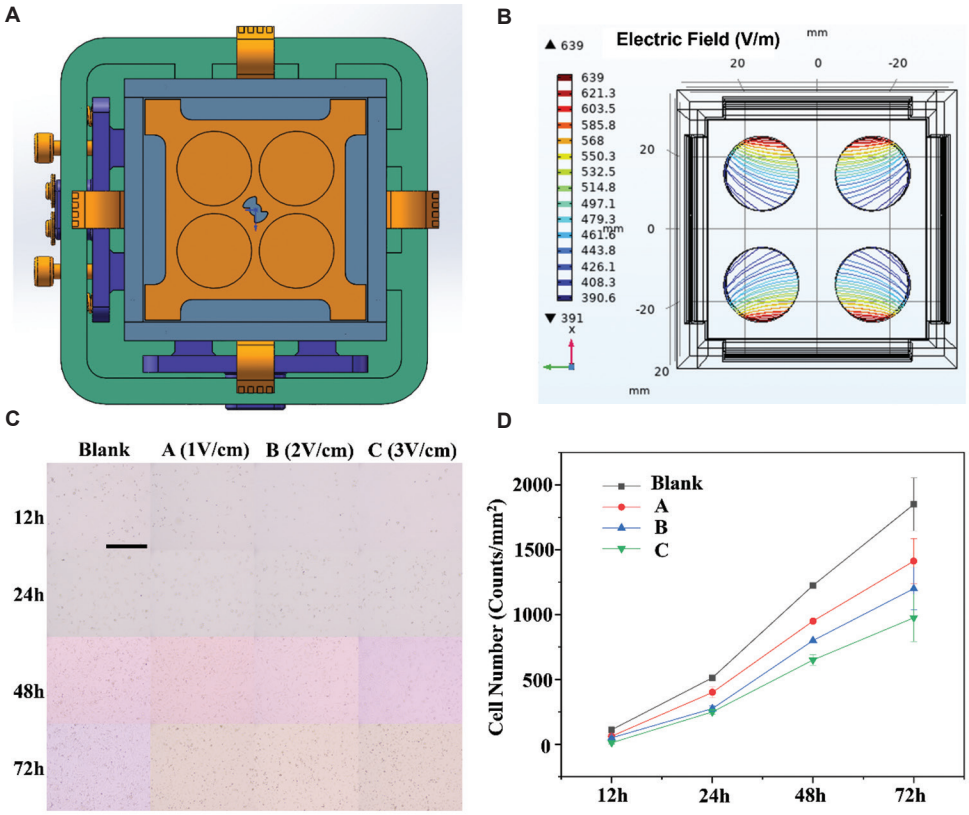


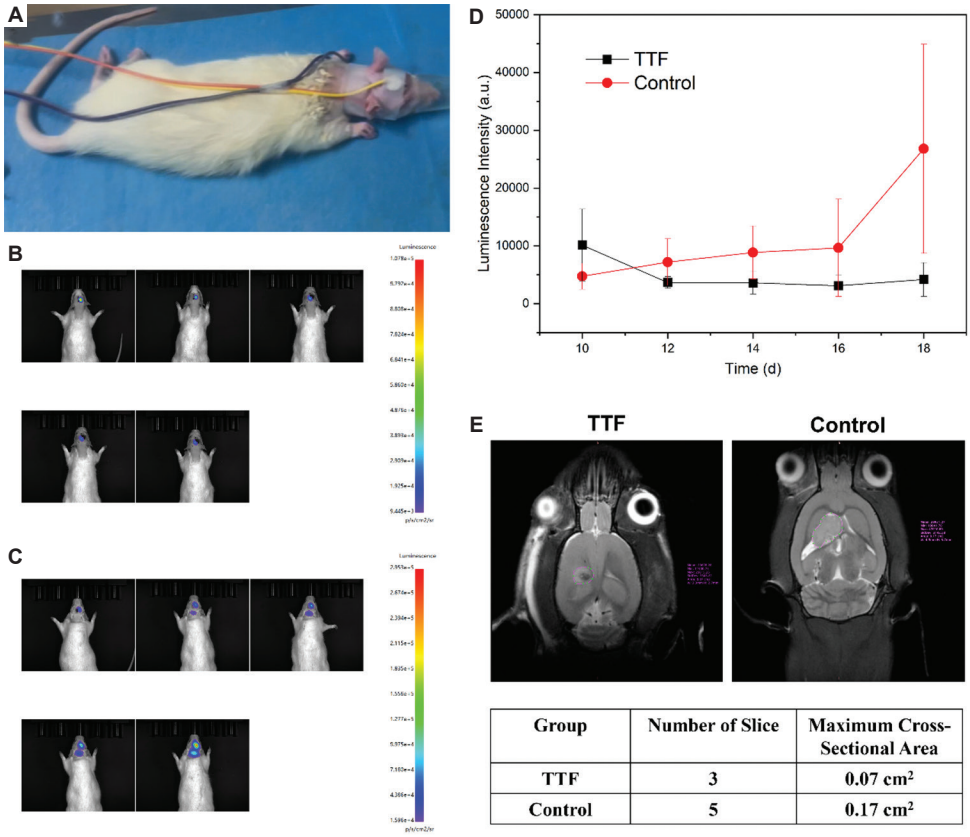
Figure 2. Cell experiments of TTF therapy. (A) The top view of the cell experiment prototype design, showing the placement of round cell culture glass slides and the orthogonal electric field application. (B) The finite element simulation of the electric field distribution using Comsol software. (C) Images of U251 cells cultured under different electric field intensities and at various time points (12, 24, 48, and 72 h). Scale bar: 0.5 mm. (D) The quantitative analysis of adherent cell counts.
Abbreviation: TTF: Tumor treating fields.

confirming the uniform application of the field over the cells.

The visual comparison of cell morphology across different electric field strengths shows a significant reduction in cell proliferation, especially at higher field intensities. Figure 2C presents micrographs of cells treated under 1, 2, and 3 V/cm electric fields at 12, 24, 48, and 72-h intervals. A marked decrease in cell number and altered cell morphology were observed at all-time points in the TTF-treated group, with the highest intensity (3 V/cm) showing the most pronounced effects. After 72 h, cell counts revealed a 35% reduction in proliferation at 2 V/cm and a 50% reduction at 3 V/cm compared to the control group (Figure 2D). These results demonstrate that the TTF system significantly inhibited glioblastoma cell growth *in vitro*. The inhibitory effect appeared to be both time- and intensity-dependent, with higher electric field intensities resulting in greater inhibition of cell proliferation.

For the *in vivo* experiments, a rat glioblastoma model was established to evaluate the efficacy of TTF therapy. The ethical approval (202404A010) from the Ethics Review Committee was obtained before the research. Figure 3A shows the placement of four electrode patches on the rat's head, positioned orthogonally to ensure comprehensive electric field coverage. C6-LUC glioblastoma cells, derived from the rat C6 glioma cell line by stably integration of a constitutive firefly luciferase expression construct, were injected into the brain, and TTF therapy was applied at 2 V/cm for >20 h/day for a period of 20 days.

Tumor growth was monitored using bioluminescence imaging at multiple time points-days 10, 12, 14, 16, and 18 post-inoculation (Figure 3B and 3C). Bioluminescence intensity served as an indicator of tumor size, with stronger signals representing larger tumors. Comparison between the TTF-treated and control groups revealed significant tumor suppression in the treated group over time (Figure 3D). By day 18, tumor luminescence intensity was reduced by more than 5 times in the TTF-treated group compared to controls. In addition, MRI scans performed on day 18 (Figure 3E) provided further validation of the tumor size reduction, with TTF-treated rats exhibiting smaller tumor



Group	Number of Slice	Maximum Cross-Sectional Area
TTF	3	0.07 cm^2
Control	5	0.17 cm^2

Figure 3. Rat experiments of TTF therapy. (A) The setup for the TTF treatment on the rat's head with 4 electrode patches placed on both sides of the head. (B and C) The bioluminescence images of rats from the TTF-treated group and the control group, respectively, over several days. (D) The comparative graph of bioluminescence intensity between the treatment and control groups. (E) MRI scans from day 18 showing reduced tumor size in TTF-treated rats compared to rats in the control group.
Abbreviations: MRI: Magnetic resonance imaging; TTF: Tumor treating fields.

cross-sectional areas compared to controls. These results demonstrate the effectiveness of the TTF system in slowing tumor progression in the glioblastoma rat model.

During TTF therapy, additional physiological parameters were monitored to assess treatment safety and tolerability in the glioblastoma rat model. Figure 4A shows the detailed process of electrode placement on the rats, ensuring proper alignment for optimal electric field delivery. Body weight changes were tracked throughout the experiment, as shown in Figure 4B, indicating that TTF-treated rats maintained a relatively stable body weight compared to the control group, suggesting minimal adverse effects on overall health. To assess potential thermal damage from the electrode patches, temperature measurements were recorded at the electrode-skin interface during TTF treatment (Figure 4C). The data showed no significant increase in skin temperature over time, confirming that the treatment did not induce thermal injury. However, some mild skin damage was observed at the electrode attachment sites on days 8, 10, and 12 (Figure 4D), where local redness and slight erosion occurred. These skin lesions were manageable and did not progress to severe injury, demonstrating the favorable overall safety profile of the TTF system despite some localized effects.

4. Discussion

The present study successfully developed and validated a novel TTF system for GBM therapy, demonstrating significant efficacy in both *in vitro* and *in vivo* models. Our findings contribute to the growing body of research supporting the therapeutic potential of TTF while also introducing innovative elements in device design, electric field optimization, and biological impact assessment. In this section, we discuss the implications of our findings, the limitations of the study, and potential future directions for improving TTF therapy in clinical applications.

4.1. Mechanism of action and optimization

Our TTF system operates at a frequency of 200 kHz and an intensity of 2 V/cm, which are consistent with clinically

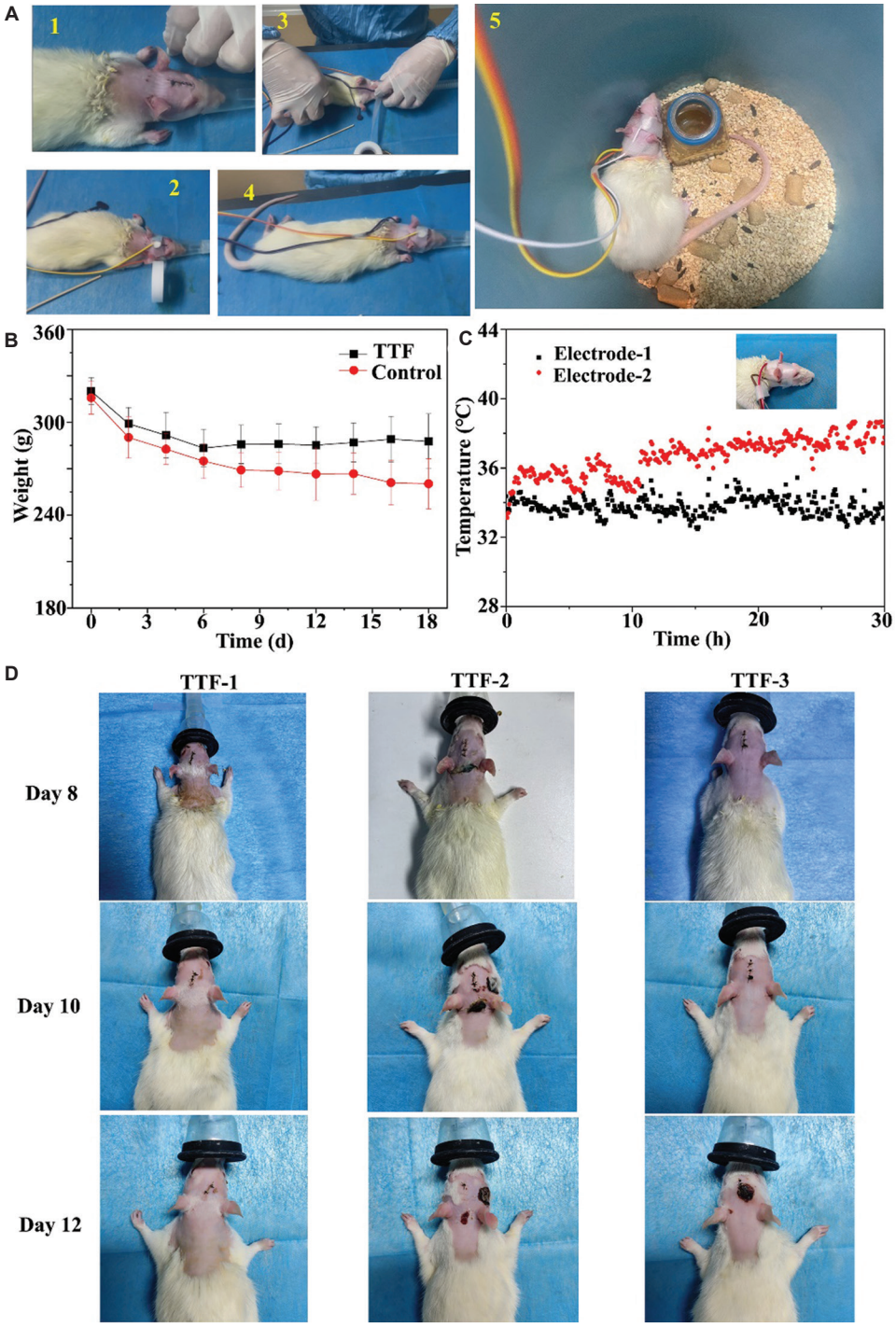


Figure 4. Safety of TTF therapy. (A) The electrode installation process and treatment setup for rats undergoing TTF therapy. (B) The change in body weight over time for both control and TTF-treated groups. (C) The temperature profile at the electrode-skin interface during TTF therapy. (D) The skin condition of rats in the treatment group on days 8, 10, and 12.
Abbreviation: TTF: Tumor treating fields.

approved TTF parameters. Our device incorporates high-dielectric ceramic electrodes made of barium titanate zirconate, which significantly improves electric field transmission and uniformity. These improvements are crucial as uniform and precise electric field distribution enhances therapeutic efficacy while minimizing unwanted side effects. Furthermore, the modular design of our control system allows independent manipulation of electric field

parameters, offering the flexibility to optimize treatment for individual patients or different tumor types.

The observed inhibition of glioblastoma cell proliferation and tumor progression in our models aligns with previous research indicating that alternating electric fields disrupt mitotic spindle formation, interfere with tubulin polymerization, and induce apoptosis in rapidly dividing cancer cells. Importantly, the *in vitro* findings demonstrated a dose-dependent response, with higher electric field intensities leading to greater tumor suppression. This suggests the potential for further optimizing field strength and exposure duration to maximize efficacy while maintaining safety.

4.2. Comparison with existing TTF devices

At present, the FDA-approved Optune system represents the benchmark for TTF therapy. However, it is limited by its relatively static field distribution and reliance on external wearable electrodes. Our system's integration of high-dielectric ceramic electrodes addresses these limitations by ensuring more efficient electric field penetration and a more homogenous distribution over tumor-affected areas. In addition, our system's control module enables dynamic field modulation, which could provide greater adaptability in treating heterogeneous and irregularly shaped tumors.

Moreover, while the Optune system has demonstrated survival benefits when combined with temozolomide chemotherapy, its efficacy remains suboptimal for some patient populations. Our system presents an opportunity to explore combination therapies, such as pairing TTF with immunotherapies, targeted inhibitors, or gene therapies. This combinatorial approach could further enhance treatment outcomes and extend survival benefits beyond what is currently achievable with standard TTF therapy.

4.3. Safety and tolerability considerations

One of the primary concerns associated with TTF therapy is the potential for adverse effects such as skin irritation and discomfort at electrode attachment sites. Our study monitored physiological responses in rat models and observed only mild skin irritation, which did not progress to severe tissue damage. This suggests that our electrode design minimizes localized heating and mechanical stress. Future research should focus on developing bioadaptive materials that further reduce skin irritation and improve patient compliance.

In addition, concerns regarding TTF's effects on normal brain tissue and neural function remain largely unexplored. While TTF selectively targets rapidly dividing cells, understanding its long-term impact on the surrounding neural microenvironment is essential. Investigating whether TTF influences neuroinflammation, neuronal plasticity, or cognitive function could inform strategies to enhance safety and mitigate any potential neurotoxicity.

4.4. Future directions and clinical translation

Despite promising preclinical results, translation of our newly developed TTF system into clinical practice requires further experimental validation. Future studies should include large-scale animal models with longer treatment durations to assess long-term efficacy and safety. In addition, clinical trials will be necessary to determine whether the advantages observed in our preclinical models translate into meaningful benefits for GBM patients.

One promising direction is the integration of real-time monitoring and feedback control in TTF therapy. Advanced sensor technologies and machine learning algorithms could be employed to personalize treatment based on tumor response dynamics. This precision medicine approach could optimize electric field parameters in real time, maximizing therapeutic effects while minimizing unnecessary exposure.

Another avenue for exploration is the combination of TTF therapy with other modalities such as hyperthermia, photodynamic therapy, and nanomedicine-based drug delivery. These synergies may enhance tumor sensitivity to TTF while overcoming some of the resistance mechanisms associated with conventional treatments. In addition, investigating TTF's applicability to other aggressive cancers, such as pancreatic or lung cancers, could broaden its clinical impact.

5. Conclusion

Our study presents a comprehensive and systematic approach to the development and evaluation of an advanced TTF therapy system for GBM. By incorporating high-dielectric ceramic electrodes and a modular control system, we demonstrated improvements in electric field transmission and therapeutic efficacy compared to existing TTF devices. The preclinical results showed that our system effectively inhibited glioblastoma cell proliferation *in vitro* and reduced tumor growth *in vivo*, supporting its potential as a novel approach for GBM treatment.

Importantly, our study also highlighted the need for further validation of the therapy system to establish its clinical significance. While our device demonstrated promising results in preclinical models, additional studies are required to assess its comparative efficacy, safety profile, and ease of integration into standard treatment regimens. The findings suggest that while improved electrode materials and field modulation strategies may offer advantages, these benefits must be rigorously tested in larger, more diverse experimental models.

Looking ahead, future research should focus on optimizing electric field parameters, exploring combination therapies, and integrating real-time monitoring for adaptive treatment strategies. Clinical translation will require rigorous trials to validate the safety and effectiveness of this technology in human patients. If successfully implemented, our TTF system could represent a significant advancement in glioblastoma treatment, offering a non-invasive and highly adaptable therapeutic option for improving patient outcomes. Overall, this study contributes valuable insights into the engineering and biological aspects of TTF therapy, paving the way for further innovations in electric field-based cancer treatments.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Zhiyong Wang
Formal analysis: Han Lv
Investigation: Xindong Wang
Methodology: Xindong Wang
Writing–original draft: Xindong Wang
Writing–review & editing: Han Lv, Xian Wang

Ethics approval and consent to participate

The study is approved by Ethics Review Committee of Hangzhou Yanqu Information Technology Co., Ltd. (approval no.: 202404A010).

Consent for publication

Not applicable.

Availability of data

All datasets on which the conclusions of this paper rely have been presented in the main manuscript.

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ACCSCIENCE PUBLISHING

Tumor Discovery

ORIGINAL RESEARCH ARTICLE

The role of disulfidptosis-related genes in the clinical prognosis and immune status of hepatocellular carcinoma

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Abstract

Drug resistance and poor prognosis in hepatocellular carcinoma (HCC) underscore the urgent need for novel treatments. Disulfidptosis, a recently identified form of metabolism-related regulated cell death, plays a complex role in anti-tumor immunity; however, its precise function in HCC remains unclear. Understanding the proteins and pathways involved in disulfidptosis and its association with disulfidptosis-related genes (DRGs) in HCC could reveal innovative therapeutic strategies. This study employs bioinformatics to examine the correlation between DRGs and both clinical prognosis and immune status in HCC patients. Risk models were constructed using univariate Cox and least absolute shrinkage and selection operator regression to identify significant genes, with risk scores correlated to survival outcomes across various patient subtypes. In addition, the analysis explored the association of DRGs with prognosis, immune cell infiltration, enriched functional pathways, and immune checkpoints. The risk model identified six key genes: *FLNA*, *NCKAP1*, *CD2AP*, *RPN1*, *SLC7A11*, and *CAPKAP*. Validation through the receiver operating characteristic curve demonstrated the model's exceptional predictive power. Gene network analysis revealed ten essential genes, three of which (*FLNA*, *CD2AP*, and *CAPZB*) were shared with the risk model. *FLNA* and *CAPZB* have previously been linked to therapeutic indicators and pathways in HCC. However, there is a lack of comprehensive data connecting *CD2AP* to clinical therapy or HCC pathways. These findings highlight the significance of DRGs in HCC prognosis and immune regulation, suggesting that DRG-targeted therapies may offer new avenues for HCC treatment.

Keywords: *CD2AP*; Disulfidptosis; Hepatocellular carcinoma

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1. Introduction

Hepatocellular carcinoma (HCC) is a prevalent malignant neoplasm of the digestive system and is the most common primary liver tumor. HCC ranks sixth (4.3%) among newly diagnosed cancer cases globally and is the third (7.8%) leading cause of cancer-related deaths worldwide.[1] The annual incidence of HCC has been rising, and the

outcomes of treatment remain suboptimal, with only 15% of patients surviving for 5 years.[2] In China, liver cancer is the third most common cancer and the second leading cause of cancer-related mortality.[3] Most patients succumb to tumor progression and recurrence despite receiving aggressive treatment modalities such as chemotherapy and surgical resection.[4,5] Nevertheless, research suggests that immune checkpoint inhibitor (ICI) therapy offers novel therapeutic options for HCC by targeting various cancers.[6,7] However, studies have not identified any prognostic biomarkers for ICI therapy. Consequently, identifying reliable biomarkers for ICIs in HCC remains of utmost importance.

Disulfidptosis, a recently described form of metabolism-associated regulated cell death, was identified in February 2023 in a study by Liu *et al.*[8] It is associated with disulfide linkages between internal and external protein molecules. Disulfide stress, caused by the accumulation of disulfide-bonded substances in cancer cells with elevated *SLC7A11* expression and glucose deprivation, disrupts the normal binding of disulfide bonds between cytoskeletal proteins. This disruption induces conformational and functional alterations in proteins, ultimately leading to cell death.[8] Research has revealed that the *SLC7A11* gene plays a significant role in disulfidptosis, primarily influencing the development of specific malignancies and anti-tumor immunity. Disulfidptosis is anticipated as a potential cancer treatment, given that previous research has demonstrated the widespread expression of the *SLC7A11* gene in numerous cancer cells.[9] Disulfidptosis-related genes (DRGs) have been used to screen for prognostic markers and potential therapeutic targets in various malignancies, such as colorectal adenocarcinoma.[10] In addition, DRGs have been associated with the development of HCC.[11] Furthermore, numerous studies have demonstrated that *SLC7A11* is associated with cell death across various organs and tissues in the human body. For example, tumor growth has been promoted by the overexpression of *SLC7A11*, partly due to the suppression of ferroptosis, while inhibition of *SLC7A11* is more likely to induce autophagic death and apoptosis as a result of elevated intracellular reactive oxygen species levels.[12] The downregulation of *SLC7A11* has been shown to impair the cysteine metabolic pathway, affecting glutathione synthesis, which leads to the accumulation of lipid peroxides and ultimately causes ferroptosis cell death.[13]

Elevated *SLC7A11* expression has been found to activate the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway, promoting the migration and invasion of pancreatic cancer cells.[14] Moreover, the mechanisms of disulfidptosis have been proposed as a critical new approach to disease treatment.[15] For example, intracellular disulfidptosis has been associated with oxidative stress, and the formation of invasive pseudopods in metastatic tumors has been suggested to exacerbate disulfide bonding stress.[16] Disulfide production is regulated by several signaling pathways, including the nuclear factor kappa B and c-Jun N-terminal kinase pathways.[17] In cardiovascular diseases, cellular damage has been mitigated by inhibiting sulfur dioxide.[18] Thus, disulfidptosis is considered to hold significant potential as a therapeutic strategy. However, the role of disulfidptosis in HCC remains unclear. Hence, in this study, DRGs were identified to develop a risk score model. Genes associated with DRGs were screened using univariate Cox regression and least absolute shrinkage and selection operator (LASSO) regression analyses. A risk model was developed, and its predictive ability was assessed. The association between prognostic and clinical features was examined, followed by functional enrichment analysis and the construction of a protein-protein interaction (PPI) network. Gene set enrichment analysis (GSEA) and immune infiltration correlation analyses were performed to investigate the expression levels of immune checkpoint genes and DRGs in HCC and normal tissues. In addition, the gene expression and drug sensitivity profiles of DRG-related genes in tumors were analyzed. These signaling pathway genes have potential as therapeutic targets due to their roles as oncogenes. To elucidate the role of disulfidptosis in HCC, our study aims to explore the association between DRGs and both the immunological profiles and clinical prognosis of HCC patients.

2. Data and methods

2.1. Materials and data

2.1.1. Data collection

Clinical data for 424 patients with HCC were obtained from the Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). Data from the 424 TCGA-liver HCC samples were standardized using the "Surrogate Variable Analysis" package in R software (version R 4.4.2). Subsequently, 79 immune checkpoint-associated genes were identified from the review,[19] along with 23 differentially regulated genes (*SLC7A11*, *ACTN4*, *GYS1*, *MYH10*, *NDUFS1*, *IQGAP1*, *MYL6*, *NDUFA11*, *PDLIM1*, *NUBPL*, *NCKAP1*, *CD2AP*, *LRPPRC*, *INF2*, *SLC3A2*, *MYH9*, *RPN1*, *ACTB*, *CAPZB*, *DSTN*, *FLNA*, *FLNB*, and *TLN1*) reported by Liu *et al.*[8] The mRNA expression data were normalized using the appropriate R package. A total of 23 genes were selected based on the criteria of |logFC| > 0.5 and false discovery rate (FDR)< 0.05. Differentially expressed genes were initially identified using the "limma" package in R software, identifying 18 DRGs (Figure 1).

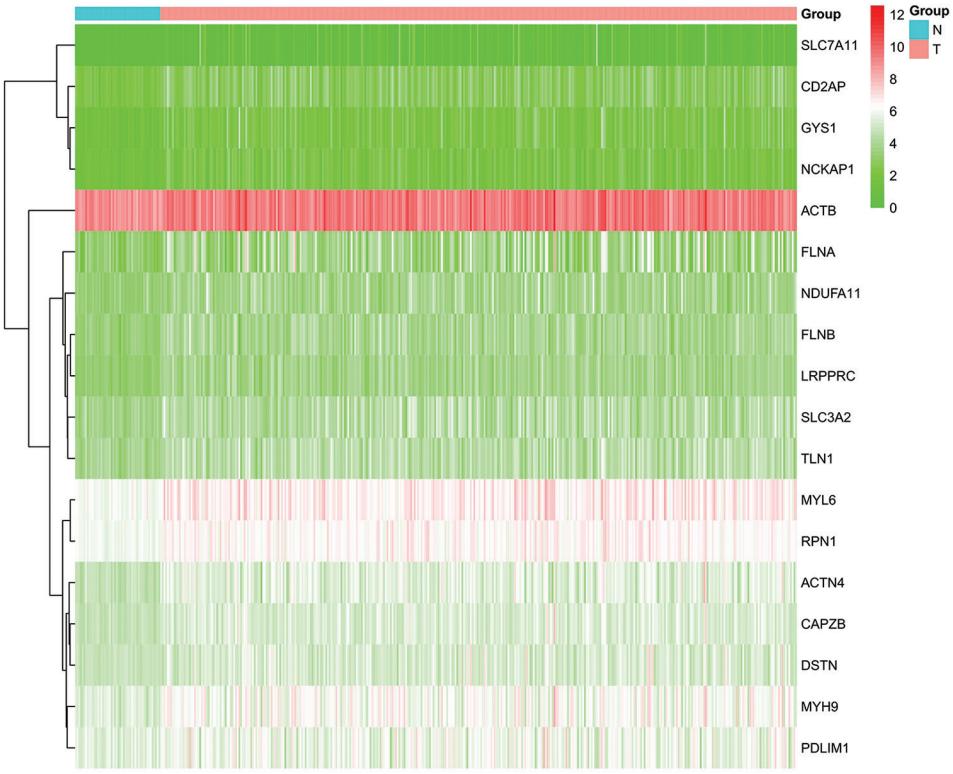

Figure 1. Heatmaps of 18 disulfidptosis-related gene expression levels. Each column represents a sample, and each row represents a gene. N refers to normal tissue, T refers to tumor tissue, red indicates high expression, and green indicates low expression.

2.1.2. Materials

Primers for *GAPDH*, *CAPZB*, *FLNA*, and *CD2AP* were designed using the Primer Bank database hosted by Harvard Medical School (Table 1). The normal human hepatocyte cell line (Lx2) and human HCC cell lines (Huh7, HepG2, and Hep3B) were acquired from the American Type Culture Collection (United States). The cells were cultured at 37°C with 5% carbon dioxide. Total RNA was extracted using TRIzol reagent, followed by complementary DNA (cDNA) synthesis using a cDNA synthesis kit from TaKaRa (United States). Quantitative polymerase chain reaction (qPCR) was performed using ChamQ SYBR qPCR Master Mix (Vazyme, China).

Table 1. Gene and primer sequence

Gene name		Primer sequence (5' – 3')
GAPDH	Forward	CATGAGAAGTATGACAACAGCCT
	Reverse	AGTCCTTCCACGATACCAAAGT
FLNA	Forward	GTCACGGGCTAGGTGCTG
	Reverse	GTCCACATCCACCTCTGAGC
CAPZB	Forward	CCCAGCAAATCGAGAAAAACCT
	Reverse	CAAGGGAGGGTCATACTTGTTAC
CD2AP	Forward	GGCATGGGAATGTAGCAAGTC
	Reverse	CCACCAGCCTTCTTCTACCTC

2.2. Construction and validation of a prognostic model based on DRGs

Univariate Cox regression analysis was employed to evaluate the prognostic value of DRGs. The prognostic risk model was developed using the "survival," "survminer," and "glmnet" packages in R software. Regression coefficients and risk scores were then calculated to establish both risk score and clinical factor models. The risk score formula was defined as follows in Equation I.

$$\text{Risk score} = (\text{Coefficient of regression}_1 \times \text{mRNA expression}_1 + \text{coefficient of regression}_2 \times \text{mRNA expression}_2 + \ldots + \text{coefficient of regression}_n \times \text{mRNA expression}_n \quad \text{(I)}$$

The LASSO-Cox regression model for mRNA was validated by the regression coefficients. HCC patients were stratified into low-risk and high-risk groups based on the median risk score. LASSO regression analysis was utilized to refine the selection of DRGs further. The survival prognosis of the two patient subgroups was evaluated using Kaplan-Meier curves. The mean risk scores were calculated from all HCC samples. Patients with HCC were

categorized into high-risk (≥mean) and low-risk (<mean) groups based on these mean risk scores. Survival curves for these subgroups were then plotted. The "survminer" package in R software generated Kaplan-Meier curves, risk line plots, and scatterplots. These visualizations were used to compare risk score variations among patient subtypes with DRGs and to illustrate the relationship between risk levels and patient survival status. Receiver operating characteristic (ROC) curve analysis, including survival curves at 1, 3, and 5 years, was performed using the "survminer" and "timeROC" packages in R software to assess the prognostic power of the risk model. In addition, risk scores and clinical factors were integrated to construct univariate and multivariate Cox regression models, which were used to evaluate the independent prognostic value of risk scores for HCC patients.

2.3. Analysis of the correlation between prognostic and clinical features

Risk scores and clinical data were integrated and analyzed using the chi-square test to examine the correlation between prognostic and clinical features. The chi-square test was employed to investigate the involvement of prognostic characteristics in the development of HCC. Heatmaps displaying DRGs with clinic-specific data were generated. Box plots based on risk scores were created to analyze the variance in clinical factors and the correlation between prognostic and clinical characteristics. Variables such as age, sex, pathologic N, pathologic M, pathological stage, and pathologic T were considered to determine statistical significance across subgroups. Pathologic T, N, and M refer to tumor, node, and metastasis, respectively. In addition, further stratified analyses were performed to assess the prognostic significance of DRG characteristics within subgroups categorized by age (≤65 years vs. >65 years), sex (male vs. female), pathologic stage (stages I – II vs. stages III – IV), tumor grade (low vs. high), pathologic T (stages T1 – T2 vs. stages T3 – T4), and pathologic N (N0 vs. N1–2–3).

2.4. Nomogram establishment based on risk score and clinical variables

Univariate and multivariate Cox regression analyses and additional clinical variables were incorporated to assess whether risk scores possess independent prognostic value in predicting outcomes. Column-line plots were generated to evaluate the predicted likelihood of overall survival (OS). Clinical variables and risk scores were collected, and Cox regression modeling was applied to calculate hazard ratios (HRs) for each variable. The relationships between DRG-based and clinical variables were examined. Column-line plots were constructed using clinical factors and DRG-based risk ratings to evaluate the likelihood of 1-, 3-, and 5-year OS in patients with HCC. Consistency indices and a calibration curve were employed to assess the predictive accuracy of the nomogram.

2.5. Functional enrichment analyses and protein-protein interaction

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were employed to investigate differences in gene functions and pathways between subgroups identified by the risk model. Eighteen DRGs were identified between the low- and high-risk groups in the TCGA dataset. To better understand the biological pathways and functions of the identified differentially expressed genes, GO enrichment analysis (molecular function [MF], biological process [BP], and cellular component [CC]) and KEGG pathway analysis were conducted using the "ClusterProfiler," "org.Hs.eg.db," "enrichplot," and "ggplot2" packages of R software. A $p<0.05$ was considered indicative of significant enrichment.

Protein-protein interactions (PPIs) were visualized by submitting differentially expressed DRGs to the STRING database (http://www.string-db.org/). The most significant modules (the top ten highest-rated genes) within the PPI network were selected using the maximal clique centrality algorithm through the "CytoHubba" plugin in the Cytoscape software (version 3.8.0).

2.6. GSEA

To investigate the potential molecular mechanisms distinguishing the low-risk and high-risk groups, GSEA was performed using the "ClusterProfiler," "enrichplot," "ggplot2," and "org.Hs.eg.db" packages of the R software. A $p<0.05$ was considered indicative of significant enrichment. Pathways with the top five highest numbers of molecules in the gene set were selected for mapping.

2.7. The relationship between prognostic signatures and immune checkpoints

Given the significance of ICI immunotherapy, the association between two subgroups of HCC patients, categorized by risk scores and 79 immune checkpoints, was investigated, and the differences in immune checkpoint expression between these subgroups were assessed. Gene expression associated with immune checkpoints was analyzed using the "limma" and "ggpubr" packages in the R software, focusing on the differences between the high-risk and low-risk groups.

2.8. Correlation analysis between genes and immune infiltration

Immune infiltration refers to the distribution and activity of immune cells and related molecules within tumor

tissues, which is crucial for elucidating tumorigenesis, progression, and therapeutic outcomes. To investigate the relationship between DRG expression and immune cell infiltration, we submitted differentially expressed DRGs to the database (https://cistrome.shinyapps.io/timer/) to acquire information regarding the association between genes and immune cells. A higher correlation coefficient (Cor) indicates a stronger relationship between genes and immune cell infiltration.

2.9. Drug sensitivity analysis

Six DRGs were submitted to the Genomics of Drug Sensitivity in Cancer (GDSC) and the Cancer Therapeutics Response Portal (CTRP) through the Gene Set Cancer Analysis (GSCA) website (http://bioinfo.life.hust.edu.cn/GSCA/#/), which facilitated a more in-depth analysis of the relationship between DRG expression and drug sensitivity in HCC.

3. Results

3.1. Construction and validation of a prognostic model based on DRGs

3.1.1. Heatmap of gene expression levels between normal and tumor tissue

Based on |logFC| >0.5 and a FDR threshold of <0.05, 23 known genes were initially screened. Subsequently, the "limma" package in the R software was utilized to identify genes with differential expression. Through this analysis, 18 DRGs, including *SLC7A11*, *PDLIM1*, *GYS1*, *ACTN4*, *NDUFA11*, *NCKAP1*, *FLNB*, *MYH9*, *MYL6*, *LRPPRC*, *SLC3A2*, *FLNA*, *CD2AP*, *RPN1*, *ACTB*, *CAPZB*, *DSTN*, and *TLN1*, were identified. A heatmap was generated using clinical information from HCC patients to display the expression levels of the 18 genes in both normal and tumor tissues. Analysis of the left dendrogram of the heatmap revealed that *SLC7A11*, *CD2AP*, *GYS1*, and *NCKAP1* exhibited high similarity and were minimally expressed in normal tissues. In addition, the relative expression levels of *ACTB* were higher in both normal and tumor tissues. The expression levels of *MYL6* and *RPN1* were upregulated in most tumor tissues. In contrast, *FLNA*, *ACTN4*, *CAPZB*, and *DSTN* were downregulated in some tumor tissues (Figure 1). The heatmap visually represents specific gene expression (the left dendrogram indicates gene clustering, and the color blocks reflect relative gene expression), facilitating further analysis.

3.1.2. Univariate Cox regression and LASSO regression

Univariate Cox regression analysis was employed to assess the prognostic significance of HCC. It was found that only eight genes (*CAPZB*, *RPN1*, *SLC7A11*, *FLNA*, *NCKAP1*, *CD2AP*, *ACTB*, and *ACTN4*) exhibited significant prognostic value (threshold of $p<0.01$, HR > 1) (Figure 2A). LASSO regression analysis was utilized to select the optimal parameter (Lambda) for the model. Cross-validation was performed by selecting one standard error of the Lambda value, resulting in the most optimal

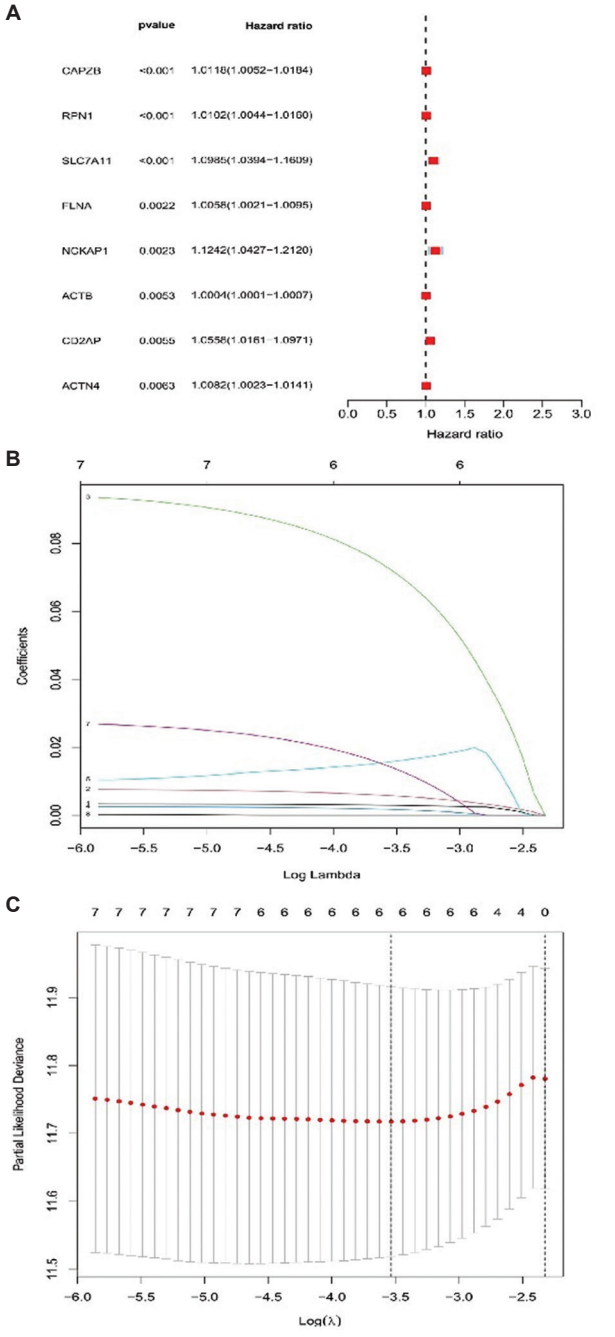

Figure 2. Correlation graphs of univariate Cox regression and LASSO regression. (A) Forest map. (B) LASSO coefficient path diagram. (C) Cross-validation used in the LASSO regression.
Abbreviation: LASSO: Least absolute shrinkage and selection operator.

model value (λ = 0.082469) (Figure 2B and 2C). This process successfully identified six genes (*CAPZB*, *RPN1*, *SLC7A11*, *FLNA*, *NCKAP1*, and *CD2AP*) (Table 2). The six meticulously screened genes underwent regression analysis to compute their respective coefficients and risk scores. Based on the median risk score, these scores were subsequently used to establish a risk model that divided HCC patients into two distinct subgroups: low-risk and high-risk groups. Comparative analysis revealed that the high-risk group, initially comprising 182 individuals, exhibited a significantly lower survival rate than the low-risk group, with 183 patients. Specifically, approximately 140 patients in the low-risk group survived after 1 year, whereas only about 114 patients in the high-risk group survived. Similarly, the survival numbers were 15 and 13, respectively, after 5 years. This stark contrast indicated a statistically significant difference (p<0.001), suggesting a higher mortality rate in the high-risk group (Figure 3A). Consequently, the high-risk group displayed a significantly greater number of deaths and a shorter OS time (Figure 3D). These findings indicated a negative correlation between risk scores and prognosis, implying that a higher risk score is associated with poorer prognosis (Figure 3A-D).

Table 2. Gene list and coefficients

Gene	Coefficients
CAPZB	0.00295
RPN1	0.00575
SLC7A11	0.07195
FLNA	0.00173
NCKAP1	0.01584
CD2AP	0.01404

Time-dependent ROC analysis was performed to assess the prognostic model's reliability. The results showed notable prognostic accuracy, with the area under the curve values of 0.727 for a 1-year prognosis, 0.676 for a 3-year prognosis, and 0.635 for a 5-year prognosis. These findings underscore the model's ability to predict prognosis precisely (Figure 3B). Furthermore, heatmap visualization was used to highlight the differential expression patterns of DRGs between the high-risk and low-risk groups within the TCGA cohort. Notably, six key genes – *CAPZB*, *RPN1*, *SLC7A11*, *FLNA*, *NCKAP1*, and *CD2AP* – exhibited distinct expression profiles. In the high-risk group, these genes showed predominantly elevated expression levels, as

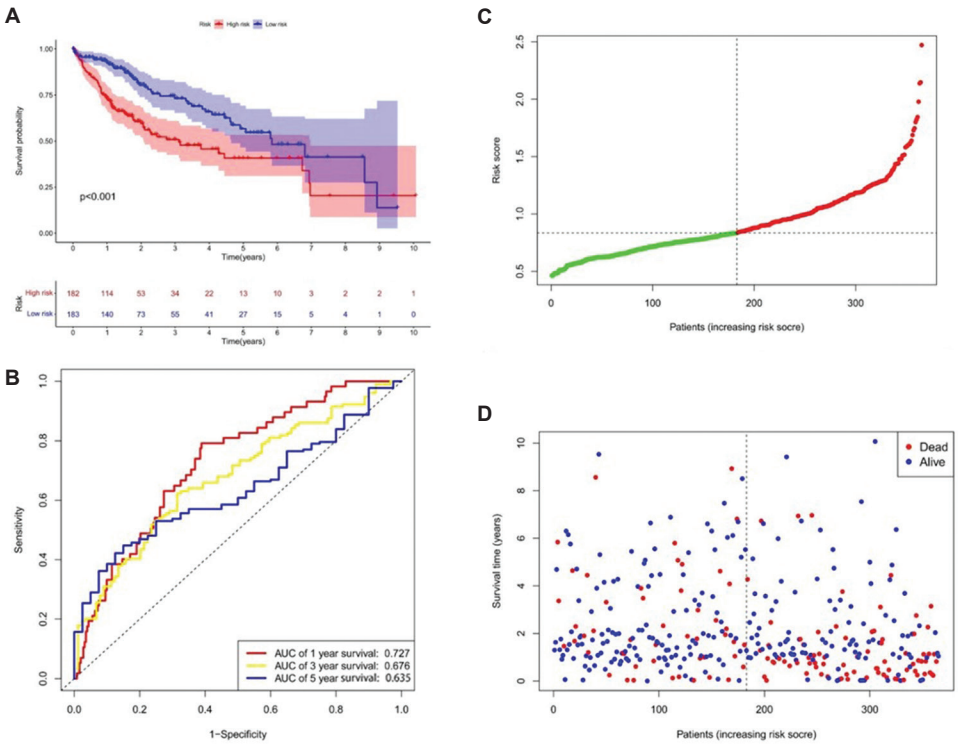

Figure 3. Construction of the prognostic disulfidptosis-related genes-based signature. (A) Kaplan-Meier survival analysis of hepatocellular carcinoma patients between high-risk and low-risk groups (B) Time-independent receiver operating characteristic analysis of risk scores predicting overall survival. (C) Risk line plot (low risk indicated by green and high risk indicated by red. (D) Risk scatterplot.
Abbreviation: AUC: Area under the curve.

indicated by the predominance of red and dark red patches. Conversely, in the low-risk group, their expression was minimal or negligible, as indicated by the predominance of black and dark green patches. This analysis depicts the genetic signatures associated with different risk levels in HCC patients, offering insights into potential therapeutic targets and prognostic biomarkers (Figure S1).

3.1.3. Correlations between the risk score and clinicopathological factors

Univariate Cox regression analysis showed that both the pathologic stage and risk score were significantly associated with the survival of HCC patients (p<0.001) (Figure 4A). Furthermore, multivariate Cox regression analysis indicated a significant association with both the risk score and pathologic stage (p<0.001) (Figure 4B). These findings suggest that DRG-based characterization serves as an independent predictive factor for HCC patients.

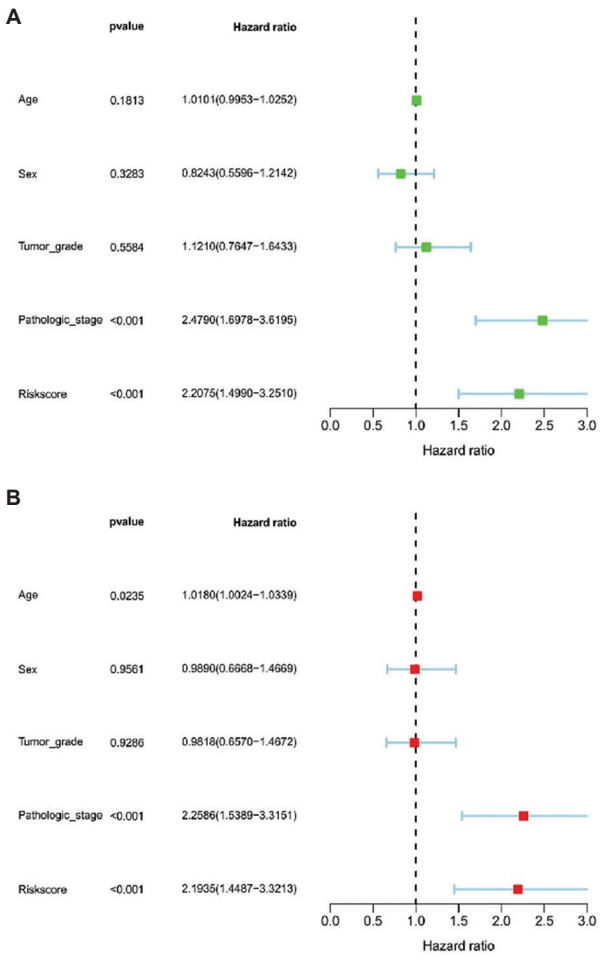

Figure 4. Modeling of risk score correlations with clinical factors. (A) Forest plot for univariate Cox regression analysis. (B) Forest plot for multivariate Cox regression analysis.

Multivariate analysis confirmed that the DRG-based risk score and pathologic stage independently predicted HCC prognosis. While age was included in the model, its association with survival outcomes was not statistically significant, suggesting that the DRG signature and tumor stage are more critical determinants of prognosis in this cohort.

3.2. Correlation between prognostic features and clinical features

3.2.1. Nomogram and calibration curve

The chi-square test was employed to determine whether prognostic characteristics were associated with the development of HCC. The results revealed significant differences between the two subgroups in terms of pathologic stage (p<0.01) and pathologic T (p<0.01). In contrast, no significant differences were observed for age (p=0.171), sex (p=0.153), pathologic N (p=0.413), and pathologic M (p=0.575) (Figures S2 and S3).

In addition, stratified analysis was conducted to assess the prognostic importance of DRGs in both risk groups. The results indicated that DRG-based characteristics were significant predictors for age ≤65 years (p=0.001), age >65 years (p=0.017), female (p=0.017), male (p<0.001), stages I – II of pathologic stage (p<0.001), T1 – T2 stages of pathologic T (p<0.001), N0 of pathologic N (p<0.001), and low tumor grade (p<0.001). However, DRG-based features were less effective in predicting stages III – IV of the pathologic stage (p=0.282), T3 – T4 stages of pathologic T (p=0.234), N1–2–3 of pathologic N (p=1.000), and high tumor grade (p=0.077) (Figure S4).

A nomogram was developed to predict survival in HCC patients further, incorporating tumor grade, pathological stage, risk score, and age to estimate 1-, 3-, and 5-year survival rates (Figure 5A). The calibration curve exhibited minor deviations from the reference gray line, and the consistency indices for the nomogram were 0.9 (1 year), 0.771 (3 years), and 0.662 (5 years), indicating that the actual patient survival closely matched the predicted values (Figure 5B). These results confirm the favorable predictive ability of the nomogram.

3.3. Functional enrichment analyses and PPI

GO and KEGG enrichment analyses were performed to elucidate the potential roles of differentially expressed DRGs. The GO enrichment analysis revealed that 18 DRGs were significantly associated with the regulation of actin filament-based processes, platelet aggregation, homotypic cell-cell adhesion, platelet activation, and blood coagulation. Among these, six DRGs were explicitly

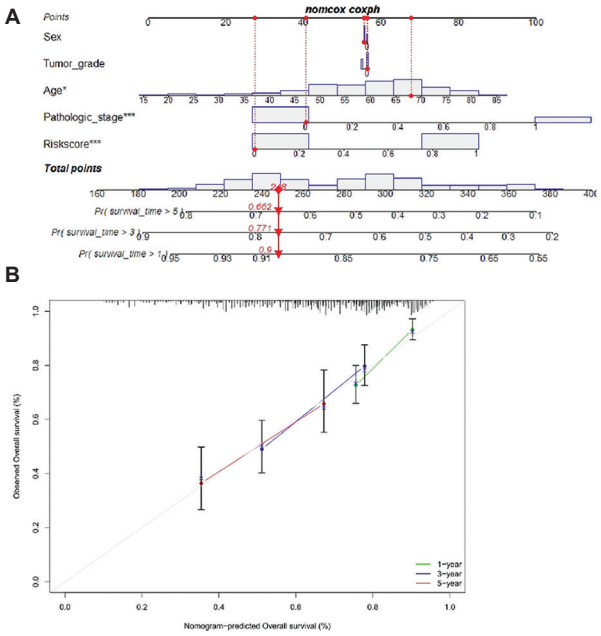

Figure 5. Correlation graphs based on risk scores and clinical variables. (A) Nomogram for predicting 1-, 3-, and 5-year overall survival. (B) The calibration curve for predicting 1-, 3-, and 5-year overall survival.
Note: *Indicates the significance of these two components.

enriched in actin filament-based processes. In addition, 18 DRGs were prominently associated with CCs such as focal adhesion, cell-substrate junction, cell cortex, brush border, and clusters of actin-based cell projections, with eight DRGs being significantly involved in each of these components. Furthermore, the 18 DRGs were notably enriched in MFs related to cadherin binding, actin binding, and actin filament binding, with eight, eight, and seven DRGs involved in each function, respectively (Figure 6A and B).

The KEGG enrichment analysis revealed that 18 DRGs were significantly involved in five metabolic pathways, namely, focal adhesion, motor proteins, tight junction, regulation of actin cytoskeleton, and *Salmonella* infection, with five, four, four, four, and four DRGs associated with each pathway, respectively (Figure 6C and D). The STRING database revealed that the PPI network of differentially expressed DRGs comprised 18 nodes and 37 edges (Figure 7A and B). Using Cytoscape software, the most important modules of the PPI network were identified, with the top 10 highest-scoring genes (*ACTN4*, *ACTB*, *CD2AP*, *CAPZB*, *DSTN*, *FLNA*, *FLNB*, *MYL6*, *MYH9*, and *TLN1*) selected (Figure 7C). The genes identified through

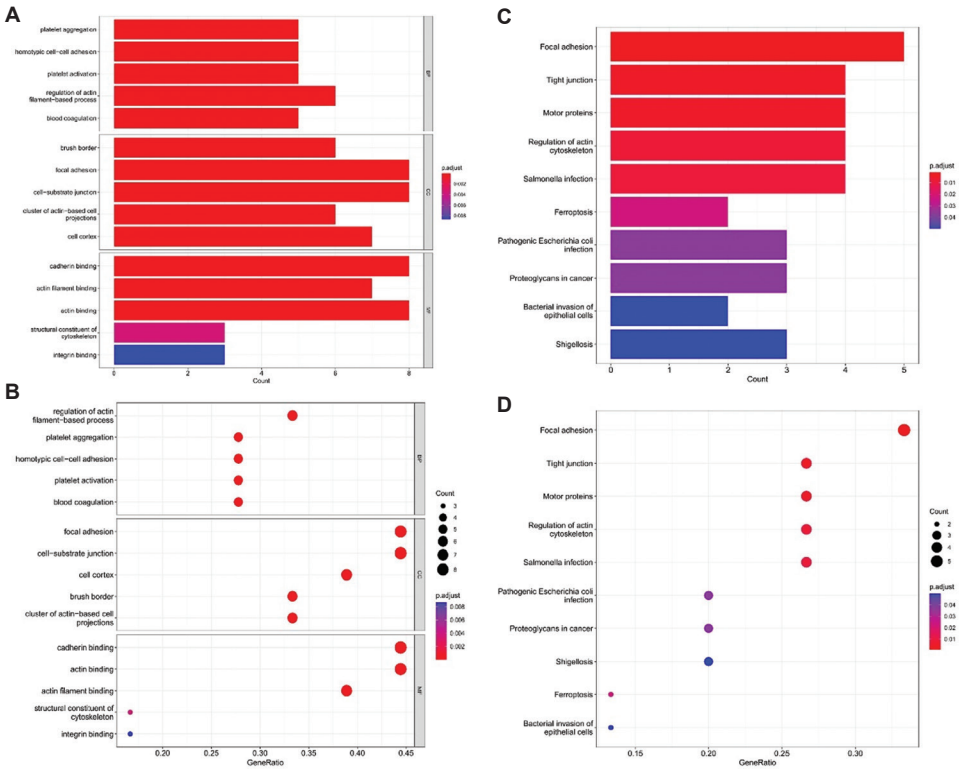

Figure 6. Enrichment analyses of disulfidptosis-related genes. (A) Gene ontology (GO) bar chart. (B) GO bubble chart (each function shows the top five pathway gene sets mainly enriched separately). (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) bar chart. (D) KEGG bubble chart.

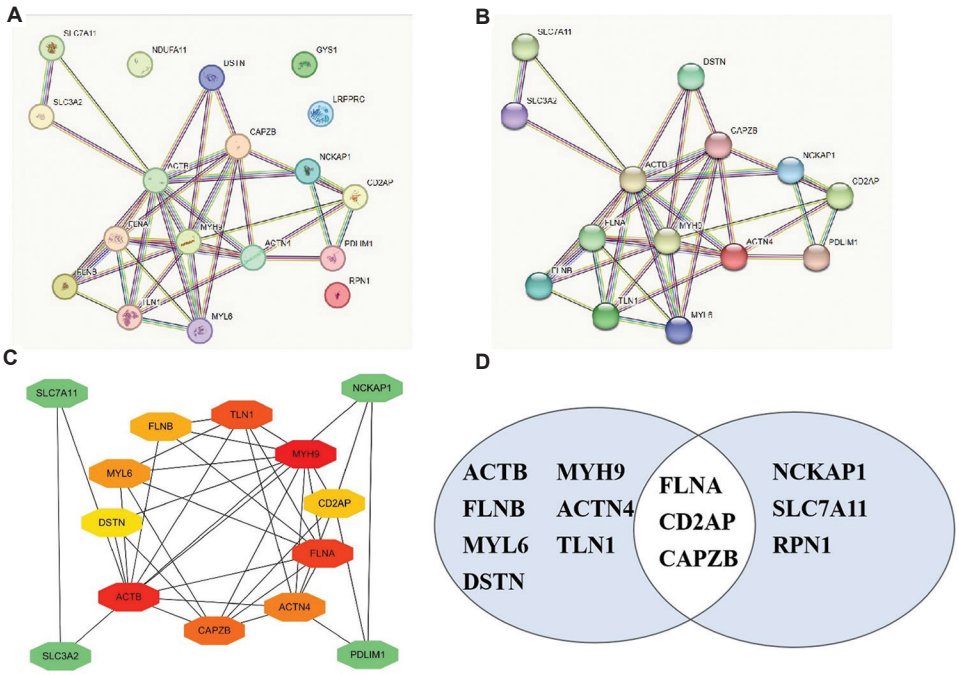

Figure 7. Diagram of the protein-protein interaction network of differentially expressed genes and diagram of the gene network. (A) Network diagram of all genes. (B) Network diagram of genes with edge and node relationships. (C) Gene network map by Cystoscope software. (D) Gene intersection map (*FLNA* and *CAPZB* have been reported to be associated with hepatocellular carcinoma, whereas no detailed hepatocellular carcinoma-related reports have been reported for the *CD2AP* gene).

this approach were compared with the risk model, and it was found that three genes (*FLNA*, *CD2AP*, and *CAPZB*) were common among the 10 genes identified through the gene network analysis, which were further refined by the six critical genes selected through regression analysis (Figure 7D). In addition, this investigation revealed that among the existing research articles related to HCC, *FLNA* and *CAPZB* have been identified as therapeutic markers and are involved in relevant pathways, while *CD2AP* has not been extensively studied in the context of HCC pathways and clinical treatment.

3.4. GSEA

The GSEA analysis was conducted to elucidate the molecular pathways underlying the characterization of DRGs. The GSEA plot results demonstrated that the enrichment scores (enrichment score value: maximum peak) were positive, indicating that the gene sets were enriched at the top of the list (Figure S5). These positive enrichment scores suggest that the DRGs were elevated in the high-risk group, with significant increases observed in efferocytosis, hepatitis C, herpes simplex virus 1 infection, oocyte meiosis, and transcriptional misregulation in cancer. The most significant effects were observed in efferocytosis and oocyte meiosis, with a corrected p=0.0096 (Table 3).

Table 3. Enrichment scores and signal pathways

Description	Set size	Enrichment score	Adjusted p-value
Herpes simplex virus 1 infection	498	0.3849	0.0380
Transcriptional misregulation in cancer	173	0.4245	0.0400
Efferocytosis	149	0.4529	0.0119
Hepatitis C	149	0.4437	0.0260
Oocyte meiosis	127	0.4689	0.0105
Taste transduction	83	0.4902	0.0107
B cell receptor signaling pathway	81	0.4875	0.0260
Acute myeloid leukemia	67	0.4902	0.0312
Cytosolic DNA-sensing pathway	64	0.5040	0.0219
N-Glycan biosynthesis	53	0.5001	0.0433
Aminoacyl-tRNA biosynthesis	25	0.5809	0.0324

3.5. Immune cell infiltration analysis

Differences in immune cell infiltration between risk groups were analyzed using immune cell infiltration analyses of the high- and low-risk groups. The results from Tumor Immune Microenvironment, CIBERSORT, CIBERSORT-ABS, quanTIseq, and Microenvironment Cell Populations-counter computational algorithms revealed that, compared to the low-risk group, the high-risk group exhibited higher

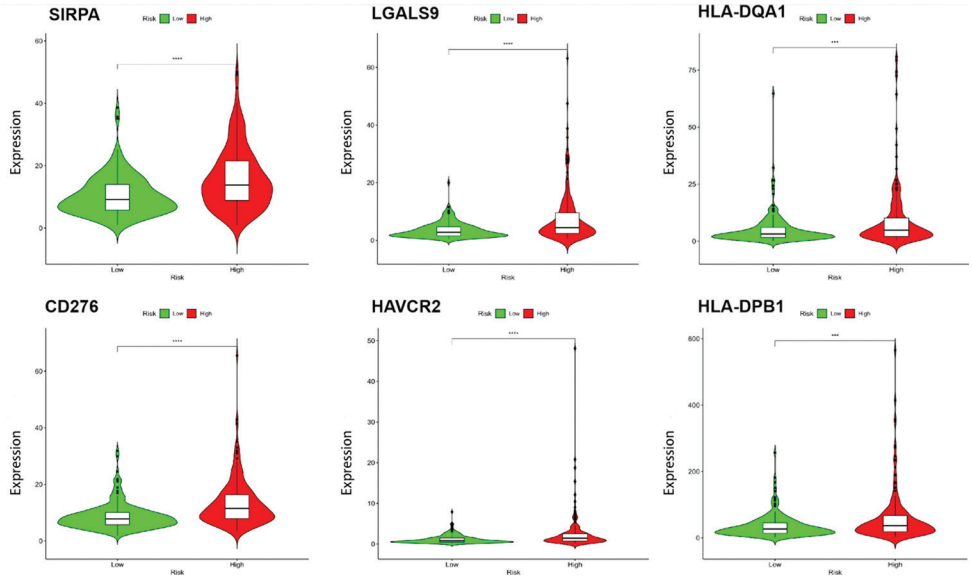

Figure 8. Violin plots indicate the relationship between prognostic signatures and immune checkpoints
Note: Statistical significance was determined at ***p<0.001 and ****p<0.0001.

levels of B cells, CD4⁺ T cells, neutrophils, macrophages, and dendritic cells. In contrast, the Estimating the Proportions of Immune and Cancer Cells algorithm showed no significant differences in immune cell levels between the high- and low-risk groups. This finding suggests that immune-related genes were more abundant in the high-risk group, exhibiting a poorer immune state (Figure S6).

3.6. The relationship between prognostic signatures and immune checkpoints

Based on risk scores, associations with 79 immunological checkpoints were investigated in two patient subgroups with HCC, and the expression of immune checkpoints between these subgroups was examined to determine whether significant differences existed. Given the importance of checkpoint inhibitor immunotherapy, the relationship between risk scores and 79 immune checkpoints was explored, with a focus on six specific checkpoints: *SIRPA*, *LGALS9*, *HLA-DQA1*, *CD276*, *HAVCR2*, and *HLA-DPB1*. The results demonstrated that all six immune checkpoint genes exhibited significantly higher expression in the high-risk group compared to the low-risk group (Figure 8). This elevation suggests the presence of immunosuppressive and exhausted phenotypes in the high-risk patient cohort.

The findings indicated that the six DRGs were most strongly associated with macrophages, neutrophils, CD4⁺ T cells, and dendritic cells (Table 4).

3.7. Drug sensitivity analysis

The results indicated relevance between the expression of six genes and the susceptibility of pan-cancer patients to the top 30 CTRP drugs. Most of these top 30 CTRP drugs exhibited positive correlations with the mRNA expression of the overexpressed genes *FLNA*, *SLC7A11*, and *NCKAP1*. Conversely, the *CD2AP* gene was negatively correlated with bosutinib, lapatinib, and austocystin D, which might enhance the sensitivity of these drugs. In addition, the overexpression of *CD2AP*, *RPN1*, and *CAPZB* genes was observed to increase sensitivity to austocystin D (Figure 9A).

In pan-cancer, relevance was identified between the expression of six genes and sensitivity to the top 30 GDSC drugs, with these highly expressed genes generally showing negative or no association with most of the drugs. The upregulation of *FLNA* was observed to lead to resistance to the small molecule drug WZ3105 while increasing sensitivity to the PI3K p110β inhibitor TGX221, midostaurin, and the HSP90 inhibitor, 17-AAG. The overexpression of *CAPZB* led to resistance to afatinib while increasing sensitivity to pazopanib and dasatinib.

Table 4. The correlation of gene and immune cells

Gene	Immune cell (Correlation coefficients: largest to smallest)		
CAPZB	Macrophage	Dendritic cell	Neutrophil
RPN1	Macrophage	CD4⁺T cell	Neutrophil
SLC7A11	Neutrophil	Dendritic cell	Macrophage
FLNA	CD4⁺T cell	Macrophage	Dendritic cell
NCKAP1	Neutrophil	Macrophage	CD4⁺T Cell
CD2AP	Macrophage	Neutrophil	CD4⁺T Cell

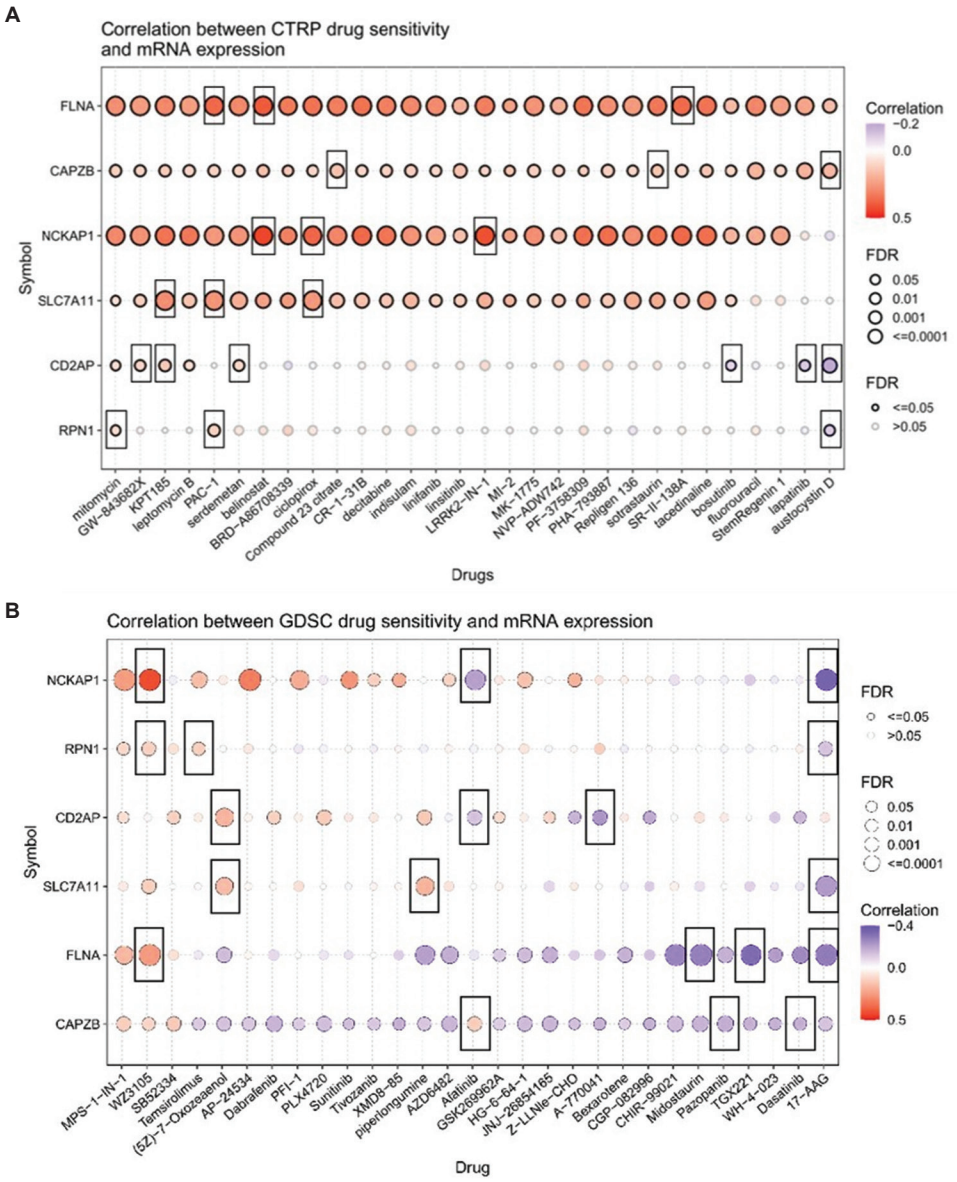

Figure 9. Correlation between six genes and the sensitivity of two database drugs in pan-cancer. (A) Cancer Therapeutics Response Portal (CTRP) drugs. (B) Genomics of Drug Sensitivity in Cancer (GDSC) drugs. The color of the bubble represents the correlation between mRNA expression and inhibitory concentration$_{50}$, and the size of the bubble is positively correlated with a false discovery rate (FDR) significance. The black outline of the bubble indicates an FDR value of <0.05. A positive correlation indicates a higher gene expression may lead to drug resistance, while a negative correlation indicates a higher gene expression may make drugs more sensitive.

In addition, the overexpression of *CD2AP* was observed to lead to resistance to the transforming growth factor-β-activated kinase 1-selective inhibitor, (5Z)-7-Oxozeaenol, while increasing sensitivity to afatinib, and the lymphocyte-specific kinase inhibitor, A-770041. Data patterns evolve, as shown in subsequent analyses (Table 5 and Figure 9B).

In summary, in the CTRP database, CD2AP, RPN1, and CAPZB overexpression increased sensitivity to sanguinarine (austocystin D). In the GDSC database, the upregulated expression of four genes (*RPN1*, *SLC7A11*, *FLNA*, and *NCKAP1*) increased sensitivity to the HSP90 inhibitor, 17-AAG. In addition, the upregulation of *RPN1*, *FLNA*, and *NCKAP1* was observed to result in resistance to the small molecule drug WZ3105. These findings may support drug-targeted therapeutic options for HCC.

3.8. Gene expression levels *in vitro*

According to the results, the expression levels of three common genes (*CAPZB*, *CD2AP*, and *FLNA*) related to

Table 5. Detailed information on the correlation between six genes and sensitivity to drugs from the databases

Gene	Drug	Correlation coefficient	False discovery rate	Correlation
CAPZB	Austocystin D	−0.141087274	0.005804727	Sensitive
	Pazopanib	−0.173849265	0.000033753	Sensitive
	Dasatinib	−0.168540986	0.005699669	Sensitive
	Compound 23 citrate	0.170831678	0.001937341	Resistance
	Sotrastaurin	0.158002623	0.000105721	Resistance
	Afatinib	0.123033433	0.0005555	Resistance
CD2AP	Austocystin D	−0.182505765	0.000015875	Sensitive
	Lapatinib	−0.110737669	0.009629998	Sensitive
	Bosutinib	−0.088728967	0.030302568	Sensitive
	Afatinib	−0.123312839	0.000537566	Sensitive
	A-770041	−0.226790986	0.000493374	Sensitive
	Kpt185	0.231873116	1.94179e-08	Resistance
	Serdemetan	0.222105917	0.168597659	Resistance
	Gw-843682x	0.217489267	2.2246e-08	Resistance
	(5z)-7-oxozeaenol	0.186845876	2.26467e-07	Resistance
FLNA	Tg×221	−0.323452482	4.56928e-09	Sensitive
	Midostaurin	−0.275955206	1.04e-13	Sensitive
	17-AAG	−0.284633046	5.11983e-16	Sensitive
	Belinostat	0.401546268	7.8603e-15	Resistance
	Pac-1	0.371434058	1.68066e-23	Resistance
	Sr-ii-138a	0.36983117	8.11047e-26	Resistance
	Wz3105	0.254281786	5.887e-14	Resistance
NCKAP1	17-AAG	−0.344806738	1.00425e-23	Sensitive
	Afatinib	−0.201855734	4.40804E-09	Sensitive
	Belinostat	0.445100924	2.73907e-18	Resistance
	Lrrk2-in-1	0.422728526	2.33952e-23	Resistance
	Ciclopirox	0.378017857	1.90393e-26	Resistance
	Wz3105	0.431735223	0	Resistance
RPN1	Austocystin D	−0.109550994	0.014444287	Sensitive
	17-AAG	−0.121562945	0.000916013	Sensitive
	Pac-1	0.120091648	0.002415796	Resistance
	Mitomycin	0.090786827	0.019872194	Resistance
	Wz3105	0.114202068	0.001178826	Resistance
	Temsirolimus	0.120785064	0.003797259	Resistance
SLC7A11	17-AAG	−0.217447834	1.08873e-09	Sensitive
	Kpt185	0.286751228	2.40003e-11	Resistance
	Pac-1	0.270432867	1.06578e-12	Resistance
	Ciclopirox	0.260975547	7.26742e-13	Resistance
	(5z)-7-oxozeaenol	0.175243229	1.40465e-06	Resistance
	Piperlongumine	0.189664289	3.33815e-07	Resistance

DGRs and immune checkpoints in HCC cell lines were further investigated *in vitro*. The results showed that the mRNA expression levels of *CAPZB* and *CD2AP* were higher in Huh7 and Hep3B cells than in normal hepatocyte cells, and no significant difference was observed in HepG2 cells. The mRNA expression levels of *FLNA* in the three HCC

cell lines were significantly lower than those in normal hepatocyte cells (Figure 10).

4. Discussion

In recent years, the incidence of liver cancer has been on the rise,[20] with HCC constituting the majority of liver cancer diagnoses and deaths.[21] Recent studies have identified a novel form of cell death termed disulfidptosis,[8] which demonstrates functional relevance in certain malignancies.[8,22] In HCC, where chronic hepatitis B virus infection constitutes the predominant risk factor, Zhang *et al.*[23] successfully constructed a prognostic model comprising five signature genes through systematic analysis of tumor prognostic characteristics associated with disulfidptosis and differential expression patterns of DRGs. The model demonstrates robust efficacy in predicting overall patient survival outcomes. Furthermore, the investigators evaluated the therapeutic implications of these signature genes across distinct risk subgroups, revealing that all five genes contribute significantly to tumor progression. Quantitative expression profiling of these genes enabled precise stratification of patients into molecular subtypes with differential prognostic trajectories. These findings collectively suggest the feasibility of targeting DRGs as a viable immunotherapeutic strategy.

This study developed a model based on the risk scores of DRGs and clinical factors in HCC. The functions and relationships of DRGs in prognosis prediction, immune infiltration, and immune checkpoints in HCC were elucidated. These findings provide a framework for predicting clinical prognosis, immune infiltration, and responses to immunotherapy, offering novel concepts and methods for targeting immunotherapy and managing patients. Six key genes (*CAPZB*, *RPN1*, *SLC7A11*, *FLNA*, *NCKAP1*, and *CD2AP*) were identified through risk modeling, and the model's strong predictive ability was confirmed by the ROC curves. Functional analysis revealed that the DRGs in the low-risk and high-risk groups correlated with several actin-related pathways,

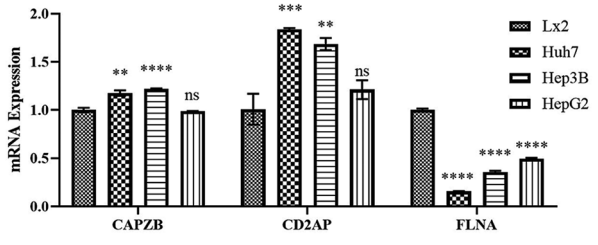

Figure 10. Expression of disulfidptosis-related genes in liver cancer cell lines

Notes: Statistical significance determined at **p*<0.01, ****p*<0.001, and *****p*<0.0001); ns indicates no significance.

with the regulation of the action network being one of the identified mechanisms of disulfidptosis.[8,13,16] Compared to the low-risk group, immune infiltrating cells were generally more prevalent in the high-risk group and were positively correlated with most immune cell types. Ten key genes were identified through gene network analysis and were compared with the risk model, with three genes (*FLNA*, *CD2AP*, and *CAPZB*) being common in both. *FLNA* and *CAPZB* have been reported to be associated with therapeutic markers of HCC. In contrast, no precise studies have been conducted on the *CD2AP* gene concerning HCC pathways or clinical treatment.

Each of these three genes has distinct functions and plays a significant role in disease research. *FLNA* has been investigated as a potential marker for HCC[24,25] and has been shown to interact with other proteins to inhibit the progression of HCC. *CAPZB* has been demonstrated in a single study to be associated with invasion and metastasis in HCC.[26] Similarly, *CD2AP* has been extensively studied in other cancers, but no published studies have reported its direct role in HCC. *FLNA* is a cytoplasmic protein with multiple functions, including interacting with actin-binding proteins to form the cytoskeleton, participating in cell motility, regulating receptor expression, interfering with signaling pathways, and playing a critical role in the development of various tumors.[27] Donadon *et al.*[28] reported that *FLNA* was expressed exclusively in HCC and not in normal liver tissue.[28] In 2021, Sheng *et al.*[29] confirmed that *FLNA* is highly expressed in HCC tissues compared to adjacent non-tumor tissues and is associated with early recurrence following resection surgery.[29] Moreover, Li *et al.*[27] reported that semaphorin 3d can regulate the invasion and metastasis of HCC cells by interacting with *FLNA*, influencing cytoskeletal remodeling, and inhibiting the PI3K/Akt signaling pathway.[27]

CD2-associated protein (CD2AP) encodes a scaffolding molecule that regulates the actin cytoskeleton. This gene has been extensively studied in other cancers, but comprehensive research on its relationship with HCC is lacking. For example, in 2016, Ren and Sheng[30] demonstrated that *CD2AP* is involved in the PI3K/Akt signaling pathway, which is implicated in cellular processes such as metabolism, proliferation, differentiation, and apoptosis.[30] In 2020, Xie *et al.*[31] found that *CD2AP* expression levels were significantly lower in diffuse gastric cancer tissues, which were associated with poor prognosis. Inhibition of *CD2AP* promoted intercellular adhesion and affected the cytoskeleton by interacting with CAPZA1, enhancing cell migration and invasion. In contrast, overexpression of *CD2AP* could attenuate metastasis in gastric cancer. Therefore, *CD2AP* may serve as a novel

biomarker associated with favorable prognosis in gastric cancer patients.[31] Chen *et al.*[32] reported that *CD2AP* can be used as a diagnostic and prognostic biomarker in patients with clear cell renal carcinoma and that DNA hypermethylation plays a significant role in reducing *CD2AP* expression.[32] In addition, *CD2AP* was upregulated in response to chronic hepatitis C virus infection, which stimulates viral transmission and steatosis by disrupting insulin signaling. Targeting *CD2AP* may offer a strategy to alleviate hepatitis C virus infection and its associated liver pathology.[33]

Therefore, *CAPZB*, *FLNA*, and *CD2AP* are anticipated to serve as novel molecular targets and offer valuable tools for assessing the effects of immunotherapy. However, this study has several limitations. For instance, data sourced from public databases were used for bioinformatics analysis, and the prediction results may be biased due to variations among different databases. Additional studies are required to validate the prediction results. In addition, the precise mechanism of *CD2A*P in HCC requires further investigation.

5. Conclusion

This study identified that DRGs are associated with the prognosis and immune status of patients with HCC. Three key genes (*CAPZB*, *FLNA*, and *CD2AP*) are crucial for further elucidating the role of DRGs in HCC. In addition, targeting differentially expressed genes may offer new approaches for diagnosing and treating HCC. Furthermore, *CD2AP* has not yet been reported to be associated with HCC pathways and clinical treatment. Hence, it may serve as a novel tumor marker and offer a potential therapeutic strategy for HCC.

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Conflict of interest

The authors declare they have no competing interests.

Author contributions

Conceptualization: Shanshan Wang
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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Publicly available datasets were analyzed in this study. Clinical data for 424 patients with HCC were obtained from the TCGA database (https://portal.gdc.cancer.gov/).

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ACCSCIENCE PUBLISHING

Tumor Discovery

ORIGINAL RESEARCH ARTICLE

Prevalence and clinical significance of rs9929218 in *Cadherin 1* and rs6983267 in the 8q24 region among Kurdish colorectal cancer patients in Iraq

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(This article belongs to the *Special Issue: Colorectal Cancer: Best Tools for Diagnosis to Management Strategies*)

Abstract

Colorectal cancer (CRC) is a leading cause of cancer morbidity and mortality worldwide, with genetic factors playing a significant role in its pathogenesis. This study investigated the prevalence of two single-nucleotide polymorphisms (SNPs) – rs9929218 in the *Cadherin 1* (*CDH1*) gene and rs6983267 in the 8q24 region – among Kurdish CRC patients in Sulaymaniyah, Iraq, and assessed their association with clinicopathological features. Blood samples from 290 CRC patients and 100 healthy controls were analyzed using allele-specific polymerase chain reaction. The frequency of rs9929218 was 20.34% in CRC patients compared to 7% in controls, while rs6983267 was detected in 26.55% of CRC cases versus 11% of controls. Both SNPs were significantly associated with CRC risk in univariate analyses; however, after adjusting for age, sex, tumor grade, and TNM stage in multivariate logistic regression, neither SNP remained an independent risk factor. Nonetheless, both SNPs showed significant associations with advanced tumor stage, nodal involvement, and perineural invasion, suggesting a potential role in disease progression rather than initiation. These findings enhance the understanding of CRC genetics in the Kurdish population and highlight the need for larger, functionally validated studies to confirm these associations.

Keywords: Colorectal cancer; Genetic polymorphism; *CDH1* gene; 8q24 region; Cancer susceptibility; Kurdish population

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1. Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality worldwide, with significant geographical and ethnic variations in its incidence and genetic predisposition. In 2020 alone, CRC accounted for approximately 10% of global cancer cases and deaths, making it the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths.[1] The identification of genetic markers associated with CRC risk has been a focal point of cancer research, aiming to enhance early detection, prevention, and personalized treatment strategies.[2] Among the numerous genetic variants studied, single-nucleotide polymorphisms (SNPs) have emerged as critical factors influencing CRC susceptibility.[3] The SNP rs9929218 in the

Cadherin 1 (*CDH1*) gene and rs6983267 in the 8q24 region are two such variants that have garnered considerable attention.[4,5]

The *CDH1* gene encodes E-cadherin, a protein essential for cell-cell adhesion and the maintenance of epithelial integrity. Mutations and polymorphisms in *CDH1* have been implicated in various cancers, including CRC, due to their role in tumor progression, invasion, and metastasis. Specifically, rs9929218 has been associated with an increased risk of colorectal adenomas and cancer. This variant may influence the expression or function of E-cadherin, thereby contributing to tumorigenesis.[6,7] As a biomarker, rs9929218 holds potential for identifying individuals at higher risk for CRC and for informing tailored prevention strategies.[8]

The 8q24 region, often described as a "gene desert," lacks protein-coding genes but contains regulatory elements that influence the expression of nearby oncogenes, such as *MYC*. The SNP rs6983267, located in this region, has been robustly linked to an elevated risk of several cancers, including CRC.[9,10] Functional studies suggest that the G allele of rs6983267 enhances the binding of transcription factors, such as TCF7L2, leading to increased MYC expression and subsequent tumorigenesis.[11] In addition, rs6983267's role in long-range chromatin interactions underscores the importance of non-coding regulatory regions in cancer genetics. The association of this SNP with CRC highlights the potential for targeting non-coding elements in future therapeutic interventions.[12]

Recent studies highlight the necessity of population-specific research to enhance our understanding of the genetic epidemiology of CRC.[13-16] While polymorphisms such as rs9929218 and rs6983267 have been extensively investigated, their prevalence and impact vary across ethnic groups, and data remain limited for several populations.[15] Most genetic association studies have predominantly focused on European and East Asian populations,[16] leaving the genetic landscape of understudied groups, such as the Kurdish population, largely unexplored.

The Kurdish population, characterized by its unique genetic background and potential founder effects, presents an opportunity to investigate these associations further. Understanding the distribution and role of rs9929218 and rs6983267 among Kurdish individuals affected by CRC could provide valuable insights into the genetic underpinnings of CRC in this population and contribute to the development of tailored screening and prevention strategies. Addressing this gap through diverse, population-based research is crucial for a more comprehensive understanding of CRC susceptibility and risk stratification.

This study aims to assess the prevalence of rs9929218 in the CDH1 gene and rs6983267 in the 8q24 region among Kurdish CRC patients. By elucidating the distribution of these SNPs within a specific ethnic group, we aim to fill the existing research gap and enhance the understanding of CRC genetics. Furthermore, our findings may support the advancement of personalized medicine in oncology, leading to improved outcomes for patients across diverse populations.

2. Methods

2.1. Sample populations

As part of a self-funded project, 290 blood samples were collected from CRC patients at Hiwa Hospital in Sulaymaniyah to investigate the presence of two SNPs. In addition, 100 blood samples were obtained from healthy individuals (54 males and 46 females), aged 37 – 79 years (median age: 59). CRC cases were selected based on the availability of clinicopathological data and the presence of at least 50% tumor tissue in the biopsy specimens and tumor block. The clinicopathological characteristics of the CRC patients are presented in Table 1.

2.2. DNA extraction

DNA was extracted from 0.4 mL of blood collected in EDTA tubes using the QIAamp DNA Blood Kit (Qiagen, Germany), following the manufacturer's protocol. The extracted DNA was eluted in 100 μL of buffer and stored at −20°C until further use.

2.3. Primer design and thermal cycling conditions

For detecting both SNPs in this study, single specific primer (SSP)-polymerase chain reaction (PCR) was employed. For each SNP, two forward primers were designed – one specific for the mutant allele and another for the wild-type allele – along with a common reverse primer. This primer design strategy was applied to both SNPs. The genomic sequences of both SNPs were obtained from the National Center for Biotechnology Information[1]. Primer design was performed using Primer3 software[2]. Primer specificity was evaluated using UCSC *in silico* PCR[3] and MFEprimer-2.0[4] to exclude potential primer-dimer formations. To confirm the results, a subset of samples containing both wild-type and mutant alleles was selected for direct, bidirectional Sanger sequencing. Additional primer sets were used to amplify the SNP regions for sequencing. PCR products

[1] http://www.ncbi.nlm.nih.gov/pubmed/
[2] http://biotools.umassmed.edu/bioapps/primer3_www.cgi
[3] http://genome.ucsc.edu/cgi-bin/hgPcr?command=start
[4] http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0/index.cgi/check_dimer

Table 1. The clinicopathological features of CRC patients in the study

Variable	Classification	*n* (%)
Sex	Male	151 (56.5)
	Female	139 (43.5)
Age	Median	69 (43 – 88)
Duke's stage	A	39 (10)
	B	97 (31.7)
	C	126 (43.4)
	D	28 (7.9)
Vascular invasion	V0	149 (47.9)
	V1	101 (31.3)
	V2	40 (10.6)
Nodal stage	N0	57 (51.3)
	N1	150 (34.8)
	N2	83 (13.7)
Tumor stage	T1	48 (16.5)
	T2	37 (12.7)
	T3	138 (47.5)
	T4	67 (23.1)

Abbreviation: CRC: Colorectal cancer.

were purified using the QIAquick PCR Purification Kit (Qiagen, Netherlands) following the manufacturer's protocol. Sequencing was performed directly using the corresponding PCR primers. The resulting chromatograms were analyzed using Chromas Lite software (v2.01, Technelysium Pty Ltd, Australia) and sequence alignment was conducted using the Basic Local Alignment Search Tool (BLAST)[5] to compare the sequences with wild-type reference sequences. All primer sequences are listed in Table 2. The PCR reaction was performed in a final volume of 25 μL, containing 1× HotShot master mix (Cadama Medical, UK), 250 nM of each primer, and 20 ng template DNA. Thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 10 s.

2.4. Statistical analysis

All statistical analyses were conducted using SPSS (v.26, IBM Corporation, US). The Hardy-Weinberg equilibrium (HWE) was assessed in the control group using the Chi-square test to determine whether genotype distributions deviated from expected proportions. Associations between rs9929218 in *CDH1* and rs6983267 in 8q24 with CRC risk were evaluated using Chi-square tests and Fisher's exact tests, with odds ratios (ORs) and 95% confidence intervals (CIs) calculated to estimate the strength of associations.

For categorical variables such as sex, tumor grade, TNM stage, perineural invasion, vascular invasion, and tumor location, Chi-square tests were used to compare distributions between wild-type and mutant SNP groups. In cases where expected frequencies in any category were below 5, Fisher's exact test was used as an alternative. Continuous variables, including tumor size and age, were assessed using the Mann–Whitney U-test, as these data did not follow a normal distribution. To adjust for potential confounders, including age, sex, tumor grade, and TNM staging, logistic regression analysis was applied. All statistical tests were two-tailed, and $p<0.05$ was considered statistically significant.

3. Results

3.1. SSP-PCR and genotyping strategy

Genotyping was performed using real-time PCR followed by melt curve analysis, allowing the differentiation between wild-type and mutant alleles based on distinct melting temperatures. Homozygous wild-type and homozygous mutant samples exhibited single melting peaks, while heterozygous samples displayed two distinct peaks representing both alleles (Figures 1 and 2). To validate the accuracy of the PCR-based genotyping approach, a subset of six samples – two homozygous wild-type, two homozygous mutant, and two heterozygous – was selected for Sanger sequencing. The sequencing results were consistent with the initial genotyping, confirming the reliability of the PCR-based approach in detecting these SNPs.

3.2. Association of rs9929218 and rs6983267 SNPs with CRC

Analysis of genotype distributions revealed a higher prevalence of rs9929218 (*CDH1*) and rs6983267 (8q24) among CRC patients compared to healthy controls (Table 3). However, increased prevalence alone does not establish an independent association, even when statistically significant in univariate analyses. To address this, we applied both univariate analysis (Chi-square and Fisher's exact tests) and multivariate logistic regression adjusting for age, sex, tumor grade, and TNM stage. The logistic regression analysis did not confirm either SNP as an independent risk factor for CRC, suggesting that the observed associations may be influenced by other confounding clinicopathological variables.

3.3. Association of SNPs with CRC risk

To determine whether rs9929218 and rs6983267 independently contribute to CRC risk, logistic regression

[5] http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi

Table 2. Primer sequences for wild-type and mutant alleles of the two SNPs

SNPs	Primers (5' to 3')	Target	Location on chromosomes
rs9929218	GTTGTACAGTCATCTGCAAGCACATGTG	Outer forward	Chr16: 68786794
	ATTCAAAGGTTCTGAATTCCACACCG	Wild-type (G)	Chr16: 68787018
	ATTCAAAGGTTCTGAATTCCACACCA	Mutant (A)	Chr16: 68787018
	GGGAGAGAAATTCCAGGGGTAGTTAACA	Outer reverse	Chr16: 68787220
rs6983267	ATTAGAAAACCTGATTTCCCTTCCAGCT	Outer forward	Chr8: 127400871
	GTCCTTTGAGCTCAGCAGATGAAGGG	Wild-type (G)	Chr8: 127401035
	GTCCTTTGAGCTCAGCAGATGAAGGT	Mutant (T)	Chr8: 127401035
	TGTCTGTATACACAGCCCAGTCTAAGGC	Outer Reverse	Chr8: 127401225

Abbreviation: SNP: Single-nucleotide polymorphism.

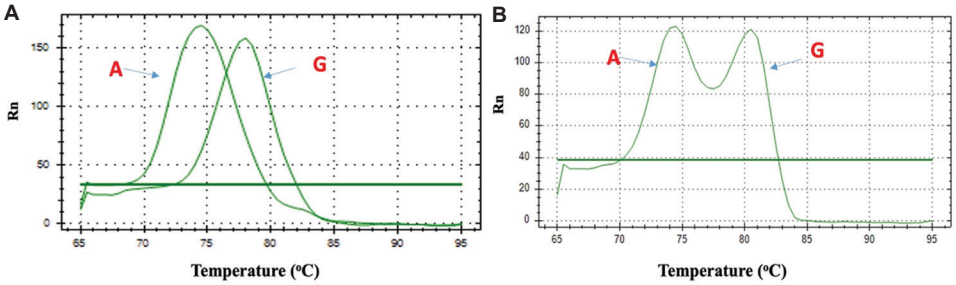

Figure 1. SNP genotyping for rs9929218 in *CDH1* by melt curve analysis using multiplex PCR. (A) Representative melt curve profiles of homozygous samples, where the wild-type allele (G) exhibits a higher melting temperature than the mutant allele (A). (B) Representative melt curve profile of heterozygous samples displaying two distinct peaks, corresponding to the wild-type (G) and mutant (A) alleles. Abbreviations: PCR: Polymerase chain reaction; SNP: Single-nucleotide polymorphism.

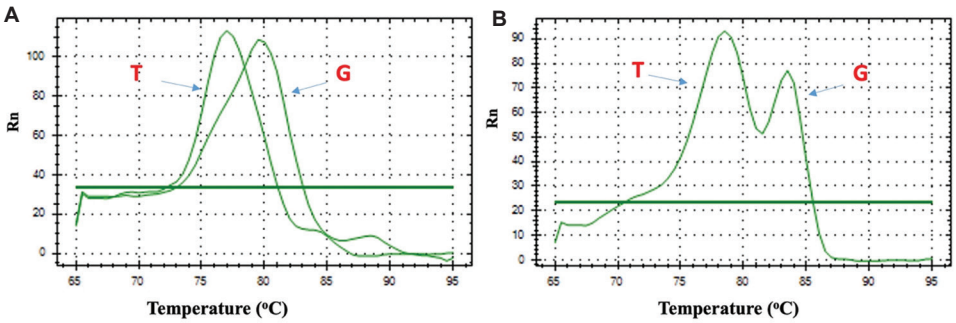

Figure 2. SNP genotyping for rs6983267 in 8q24 by melt curve analysis using multiplex PCR. (A) Representative melt curve profiles of homozygous samples, where the wild-type allele (G) exhibits a higher melting temperature than the mutant allele (T). (B) Representative melt curve profile of heterozygous samples displaying two distinct peaks, corresponding to both the wild-type (G) and mutant (T) alleles. Abbreviations: PCR: Polymerase chain reaction; SNP: Single-nucleotide polymorphism.

analysis was performed, adjusting for age, sex, tumor grade, and TNM staging. After controlling for these confounders, neither rs9929218 (p=0.194) nor rs6983267 (p=0.271) maintained statistical significance in predicting CRC susceptibility (Table 4). These findings suggest that, although these SNPs are more frequent among CRC patients, their role as independent risk factors is not supported when clinical and demographic variables are taken into account. The loss of significance after adjustment indicates that the observed associations may be influenced by tumor-related or genetic confounders.

3.4. Association of SNPs with clinicopathological features

Further analysis explored the relationship between these SNPs and various clinicopathological characteristics. Univariate analysis revealed significant associations between mutations in rs9929218 and rs6983267 and more

Table 3. Association of the two SNPs with CRC

SNP	CRC with SNP (*n*)	CRC without SNP (*n*)	Normal with SNP (*n*)	Normal without SNP (*n*)	*p* (Chi-square test)	*p* (Fisher`s Exact test)
rs9929218 (*CDH1*)	59	231	7	93	0.003	0.001
rs6983267 (8q24)	77	213	11	89	0.002	0.001

Abbreviations: CRC: colorectal cancer; SNP: Single-nucleotide polymorphism.

Table 4. Genotype and allele distributions of the two SNPs in the CRC and control groups

SNP	Genotype/allele	CRC	Control	OR (Crude)	*p* (Crude)	*p* (Logistic regression)
		n (%)	*n* (%)			
rs9929218(CDH1)	GG	231 (79.6)	93 (93)	0.836187	0.192903	0.194
	GA	53 (18.3)	6 (6)	3.115491	**0.003919***	0.194
	AA	6 (2.1)	1 (1)	2.076125	0.685527	0.686
	G	515 (88.7)	192 (96)	0.895557	0.279718	0.279
	A	65 (11.3)	8 (4)	2.876493	**0.002497***	0.279
rs6983267 (8q24)	GG	213 (73.5)	89 (89)	0.802455	0.120263	0.271
	GT	66 (22.7)	10 (10)	2.330108	**0.010653***	0.271
	TT	11 (3.8)	1 (1)	3.817235	0.316886	0.317
	G	492 (84.8)	189 (94)	0.859381	0.144846	0.144
	T	88 (15.2)	12 (6)	2.614609	**0.000944***	0.144

Notes: *$p<0.05$; Bold values indicate statistically significant results at $p<0.05$.
Abbreviations: CRC: colorectal cancer; OR: Odds ratio; PCR: Polymerase chain reaction; SNP: Single-nucleotide polymorphism.

advanced tumor stage (T3 and above) ($p<0.001$). Similarly, nodal involvement was significantly correlated with mutation status ($p=0.001$). In addition, tumors located in the colon were more frequently associated with these mutations compared to rectal tumors ($p=0.006$), suggesting a potential site-specific effect of these polymorphisms.

Multivariate logistic regression analysis, adjusted for age, sex, tumor grade, and TNM stage, confirmed that associations with tumor stage ($p=0.030$), nodal involvement ($p=0.027$), and perineural invasion ($p=0.015$) remained statistically significant. These findings suggesting a potential role of these SNPs in tumor progression, particularly in relation to advanced tumor stage (T3 and T4), rather than overall TNM stage (Table 5).

These results indicate that while rs9929218 and rs6983267 are not independent predictors of overall CRC risk, they may serve as markers of aggressive tumor behavior, particularly with respect to invasion and metastatic potential.

3.5. Genetic association analysis and HWE testing

HWE testing was conducted to evaluate whether genotype distributions deviated from expected proportions (Table 6). In CRC cases, rs9929218 showed significant deviation from HWE ($p=0.0023$), suggesting a potential role in disease susceptibility. However, the control group exhibited only a minor deviation ($p=0.043$), indicating that the variation observed in cases is unlikely to be attributed to population stratification. Similarly, rs6983267 deviated from HWE in CRC cases ($p=0.011$), while control group genotypes were closer to equilibrium ($p=0.048$).

Case-control association analysis further indicated that rs9929218 was significantly associated with CRC ($p=0.022$), whereas rs6983267 did not reach statistical significance ($p=0.090$). Due to the low frequency of homozygous mutant genotypes (AA for rs9929218 and TT for rs6983267), the analysis primarily focused on comparing heterozygotes (GA and GT) with wild-type genotypes (GG) under a dominant genetic model. However, after adjusting for clinical covariates using logistic regression, neither rs9929218 ($p=0.194$) nor rs6983267 ($p=0.271$) remained significant independent predictors of CRC risk (Table 7). Additional analysis of clinical parameters revealed significant associations between CRC progression and tumor size ($p=0.041$), tumor grade ($p=0.034$), TNM staging ($p=0.041$), and Duke stage ($p=0.038$), while vascular invasion was marginally significant ($p=0.050$) and perineural invasion did not reach statistical significance ($p=0.087$) (Table 7). To improve model accuracy, multicollinearity among clinical variables was evaluated using the Variance Inflation Factor (VIF), leading to the removal of highly correlated variables.

Table 5. Comparison of clinic-pathological features between wild-type and mutant SNPs (rs9929218 and rs6983267)

Features	Wild-type SNPs	Mutant SNPs	Test	Test statistics	*p*	*p* (multivariate logistic regression)
Age (years)	67.54% (±2.31)	65.18 (±3.07)	Mann–Whitney U-test	U=4325.5	1.0	0.890
Gender (*n*)						
Male	89	62	Chi-square	χ^2=3.24	0.072	0.112
Female	97	42				
Anatomical sites (*n*)						
Colon	136	90	Chi-square	χ^2=7.46	0.006*	0.049*
Rectum	51	13				
Tumor size (cm)	3.48 (±0.33)	5.87 (±1.06)	Mann–Whitney U	U=277	<0.001*	0.038*
Tumor grade (*n*)						
Low grade	124	50	Chi-square	χ^2=2.76	0.097	0.41
High grade	71	45				
Tumor stage (*n*)						
T2 or earlier	105	34	Chi-square	χ^2=24.55	<0.001*	0.031*
T3 or later	70	81				
Nodal stage (n)						
N0	60	11	Chi-square	χ^2=13.52	0.001*	0.027*
N1	83	30				
N2	63	43				
TNM stage (*n*)						
Stages 1 and 2	52	33	Chi-square	χ^2=0.21	0.644	0.472
Stages 3 and 4	133	72				
Perineural invasion (*n*)						
Absent	151	36	Chi-square	χ^2=45.89	<0.001*	0.015*
Present	42	61				
Vascular invasion (*n*)						
Absent	98	54	Chi-square	χ^2=0.0	1.0	0.785
Present	89	49				

Note: *p<0.05 (two-tailed significance).
Abbreviation: SNP: Single-nucleotide polymorphism.

4. Discussion

CRC is a multifactorial disease influenced by both genetic and environmental factors. Identifying genetic polymorphisms associated with CRC susceptibility is crucial for understanding disease pathogenesis and improving risk stratification. In this study, we investigated the clinical relevance of two SNPs – rs9929218 in *CDH1* and rs6983267 in the 8q24 region – among Kurdish CRC patients in Al-Sulaymaniyah province, Iraq. Our initial univariate analysis suggested a significant association between both SNPs and CRC risk, with rs9929218 detected in 20.34% of CRC cases and 7% of controls (p=0.003), and rs6983267 found in 26.55% of CRC cases and 11% of controls (p=0.002). However, after adjusting for confounding factors such as age, sex, tumor grade, and TNM staging, multivariate logistic regression did not support these associations (rs9929218: p=0.194; rs6983267: p=0.271). These findings suggest that while these SNPs may be more prevalent in CRC patients, their associations with CRC susceptibility may be confounded by other clinical variables.

The rs9929218 polymorphism in *CDH1* has been previously associated with CRC risk, particularly in European populations.[4,7] *CDH1* encodes E-cadherin, a key adhesion protein that regulates epithelial integrity, and its dysregulation is implicated in tumor progression and metastasis.[17,18] Some studies have suggested that rs9929218 may affect E-cadherin expression or function, thereby

Table 6. HWE assessment for rs9929218 and rs6983267 in CRC cases and controls

SNP	Group	Observed genotype frequencies	Expected genotype frequencies	*p*-value
rs9929218	CRC	GG: 231 GA: 53 AA: 6	GG: 228.64 GA: 57.72 AA: 3.64	0.0023
rs9929218	Control	GG: 93 GA: 6 AA: 1	GG: 92.16 GA: 7.68 AA: 0.16	0.043
rs6983267	CRC	GG: 213 GT: 66 TT: 11	GG: 208.68 GT: 74.65 TT: 6.68	0.011
rs6983267	Control	GG: 89 GT: 10 TT: 1	GG: 88.36 GT: 11.28 TT: 0.36	0.048

Notes: Expected genotype frequencies were calculated under HWE using allele frequencies derived from observed genotypes; *p*-values were computed using Fisher's Exact test.
Abbreviations: CRC: colorectal cancer; HWE: Hardy-Weinberg equilibrium; SNP: Single-nucleotide polymorphism.

Table 7. Logistic regression analysis adjusting for clinical variables.

Variable	β	SE	*p*
rs9929218	2.3173	1.783	0.194
rs6983267	−4.4398	4.032	0.271
Age	−0.4991	0.436	0.253
Tumor size	1.283	0.523	0.041
Gender (male vs. female)	0.785	0.289	0.118
Anatomical site (colon vs. rectum)	−0.914	0.421	0.089
Tumor grade (low vs. high)	1.612	0.749	0.034
Tumor stage (T2 and earlier vs. T3 and later)	2.034	0.892	0.041
Nodal invasion (present vs. absent)	−1.211	0.512	0.074
TNM stages (stages 1 and 2 vs. stages 3 and 4)	1.903	0.973	0.050
Perineural invasion (present vs. absent)	0.989	0.654	0.087
Vascular invasion (present vs. absent)	−0.632	0.419	0.102

Abbreviations: β: Regression coefficients; SE: Standard errors; SNP: Single-nucleotide polymorphism.

increasing the risk of colorectal adenomas and invasive carcinoma.[5] Our study identified a higher frequency of the A allele in CRC cases (11.3%) compared to the controls (4%), showing a significant association in the unadjusted analysis (p=0.0025). This aligns with previous research demonstrating a link between rs9929218 and increased CRC risk.[12] However, logistic regression analysis did not confirm an independent effect, suggesting that other clinical or genetic factors may contribute to CRC susceptibility in our population. This finding is consistent with a meta-analysis that reported ethnic differences in the effect size of rs9929218 on CRC risk, with inconsistent findings across diverse populations.[19]

The 8q24 region is known as a "gene desert" but harbors regulatory elements that influence the expression of oncogenes such as *MYC*, a key driver of CRC.[8] The rs6983267 SNP has been linked to an increased risk of CRC in multiple populations, particularly in individuals carrying the T allele.[11,20] Functional studies have shown that rs6983267 enhances transcription factor binding, leading to increased MYC expression and activation of Wnt signaling, both of which are critical in colorectal carcinogenesis.[21] In our study, the T allele was detected in 15.2% of CRC cases compared to 6% of controls (p=0.0009), with the GT genotype showing a higher CRC risk (p=0.0107) in the unadjusted analysis. These findings support previous reports that rs6983267 may play a role in CRC susceptibility.[22] However, as with rs9929218, logistic regression did not confirm an independent association (p=0.271), suggesting that its effect may be modulated by other genetic or environmental factors. This aligns with findings from a large genome-wide association study (GWAS), in which rs6983267 was significantly associated with CRC in unadjusted models but lost significance after accounting for confounders.[23]

Beyond CRC, the rs9929218 polymorphism has been associated with increased susceptibility to other gastrointestinal tumors, including gastric and esophageal cancers, particularly in East Asian populations.[24,25] Similarly, rs6983267 has been linked not only to CRC but also to other solid tumors such as gastric, prostate, and pancreatic cancers.[5] However, GWAS have most consistently confirmed its strong association with CRC, suggesting a tumor-type-specific regulatory role.[26] These findings indicate that while these SNPs are not exclusive to CRC, their prevalence and functional impact may vary across cancer types and populations.

CRC risk is known to vary across ethnic groups, likely due to differences in genetic background, environmental exposures, and dietary patterns.[2] The frequency of rs9929218 and rs6983267 risk alleles in the Kurdish population appears to be comparable to those reported in Middle Eastern populations but differs from European cohorts.[27] For example, a study in an Iranian cohort found a similar association between rs6983267 and CRC risk,[9] while a meta-analysis in European populations reported a higher prevalence of the T allele (up to 20%) compared to our findings (15.2%).[10] These variations highlight the importance of conducting population-specific studies to better understand CRC genetic epidemiology.

Although our study did not confirm an independent effect of rs9929218 or rs6983267 on CRC risk, these SNPs

remain potential biomarkers for genetic screening. Given their association with CRC susceptibility in previous studies, future research should explore larger multi-ethnic cohort studies to confirm whether these SNPs influence CRC risk independently or in combination with other genetic factors.[11] In addition, gene-environment interactions, including dietary patterns and inflammatory markers, may modify genetic risk. Further functional studies are needed to determine how rs9929218 and rs6983267 influence gene expression and CRC pathogenesis.[28] Moreover, integrating these SNPs into polygenic risk scores could improve risk prediction and contribute to personalized medicine.[19]

Our study has several limitations. The relatively small sample size may have reduced statistical power, and the lack of environmental and lifestyle data (e.g., diet, smoking, and physical activity) limits our ability to account for additional risk factors. Furthermore, functional validation was not performed, and future studies should investigate the biological effects of these SNPs on gene expression and tumor behavior. Despite these limitations, our findings provide valuable insights into CRC genetics in an understudied population.

5. Conclusion

This study provides insights into the prevalence and potential clinical significance of rs9929218 in *CDH1* and rs6983267 in the 8q24 region among Kurdish CRC patients. The findings indicate that these SNPs are more frequently detected in CRC cases compared to controls, suggesting a potential role in CRC susceptibility. However, after adjusting for confounding factors such as age, sex, and tumor characteristics, neither SNP remained an independent predictor of CRC risk. Despite this, significant associations were observed between these SNPs and clinicopathological features, particularly advanced tumor stage (T3 and T4) and perineural invasion, which may indicate a role in tumor progression rather than initiation. The study highlights the importance of conducting population-specific genetic research to enhance our understanding of CRC risk in underrepresented groups. Given the study's limitations, including the sample size and the absence of functional validation, further investigations involving larger, well-powered cohorts, and functional assays are needed to confirm these associations and elucidate the biological mechanisms underlying these genetic variants. Future research should also explore how these SNPs interact with environmental and lifestyle factors to refine risk stratification and contribute to personalized screening and prevention strategies for CRC.

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Conflict of interest

The author declares no conflicts of interest.

Author contributions

This is a single-authored article.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Komar University of Science and Technology (Approval number: KUST-SP25-03-01-DEN). Written informed consent was obtained from all participants before sample collection. The study adhered to the ethical principles outlined in the Declaration of Helsinki.

Consent for publication

Written informed consent was obtained from all participants for the use or publish their anonymized data in this study.

Availability of data

Data are available from the corresponding author on reasonable request.

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ACCSCIENCE PUBLISHING

Tumor Discovery

MINI-REVIEW

Exploring the cell-to-cell communication network to better defeat cancer

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Abstract

Just like us, cells communicate, but in their own unique way. Using waves as their common language, cells signal to each other about where and when to move. They talk, share information, and collaborate. The human body comprises trillions of cells that continuously adapt to their surroundings, exchanging millions of vital signals for survival. This communication must be meticulously regulated, as any disruption can lead to errors, such as the abnormal cell growth observed in cancer. The interaction between cancer cells and their neighboring cells is bidirectional, involving a complex network of mechanisms that can drive aggressive tumor behaviors—such as rapid growth, spread, and treatment resistance—or, conversely, act to suppress malignancy. This dynamic interplay within the tumor microenvironment unfolds through two primary modes: direct communication through physical cell contact, mediated by adhesion molecules, electrical signals, or the exchange of materials through gap junctions, and indirect communication facilitated by paracrine signaling. The latter involves the release of signaling molecules like cytokines, growth factors, and extracellular vesicles. Disrupting these cellular dialogues presents a promising therapeutic frontier. Specifically, strategies that integrate interventions targeting tumor communication pathways with conventional chemotherapy could enhance treatment efficacy, offering a synergistic approach to hinder cancer progression and improve outcomes. This article delves into the role of cell-to-cell communication in cancer development, its impact on metastasis, and how ongoing research is broadening our understanding of the disease.

Keywords: Cell; Communication; Cancer; Microenvironment; Metastasis

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1. Introduction

Cell-to-cell communication refers to the way cells interact to maintain homeostasis or normal functioning. It is similar to a conversation between people, where one cell sends signals or engages in physical interactions, and the receiving cell responds accordingly. For instance, a cell might express and/or secrete a peptide or a ligand, which a neighboring cell receives, interprets, and acts upon. This process is crucial for normal human tissue development and functioning, particularly in the differentiation and specialization of cells, such as stem cells.[1]

Cells communicate in various ways, such as secreting hormones that bind to receptors on neighboring cells and through direct surface contact. These signals and interactions regulate cell growth, death, and differentiation.[2] In cancer, this communication is

disrupted by cells sending out erroneous signals, creating a self-serving microenvironment that evades normal cell-to-cell cues.[3] For example, if one cell has a mutation and no longer responds to signals from another adjacent cell, it can grow uncontrollably and form a tumor. Cell-to-cell communication plays a crucial role in tumor development and clonal evolution by enabling cancer cells to reprogram the surrounding tumor microenvironment (TME) and immune cells.[4] This communication supports processes essential for tumor development and spread, such as angiogenesis, immunoediting or immunosuppression, invasion, and multi-drug resistance.

Cell-to-cell communication can occur through membrane receptors and ligands or via soluble molecules like growth factors, cytokines, and chemokines. In addition, microRNAs and extracellular vesicles have recently been identified as additional means of cell communication.[5] Circulating nucleic acids are often deregulated in many cancers and, as they affect all the disease's hallmarks, they may serve as valuable biomarkers for cancers. Meanwhile, extracellular vesicles released from cancerous cells contribute to tumor growth and distribution.

2. The community of cancer cells with their own language

Two broad themes have emerged in recent years that have changed our perspective on cancer. First, cancer cells are not only communicating with their environment and each other but also with normal cells in the body, such as cancer-associated fibroblasts or neurons. Second, cancer cells are not merely growing uncontrollably and ignoring their surroundings; instead, they are strategically coordinating actions among themselves as a community.

Unlike the relatively slow, soluble signals produced by ligands released from a cell towards a receptor over a short distance, electrical pulses might offer a rapid and distant transmission system, similar to quorum sensing in bacteria and electrical signals used by fungi. It is conceivable that similar quorum sensing systems exist in communities of cancer cells, allowing them to communicate about nutrient locations and coordinate their metabolism. Once predictable classes of communication signals are identified, we can start to see the building blocks of a code or language, where different patterns might be interpreted differently by various cells (Figure 1). Disrupting these signals could dysregulate the community, much like an army without its general. The challenge lies in understanding the language but not yet knowing the cellular response.

As tumors progress, cancer cells acquire hallmark capabilities that enable unchecked malignancy. These include autonomous growth (not triggered by external growth signals), tissue invasion and metastasis, angiogenesis induction, and evasion of proliferative restraints like apoptosis and senescence. These traits stem from dysregulated signaling pathways that normally regulate cell division, survival, and motility in healthy tissues. Many experimental cancer therapies now focus on targeting these aberrant signaling molecules — key drivers of oncogenic behavior.

Emerging research highlights that specialized cell-cell adhesion complexes, critical for maintaining epithelial polarity and tissue structure, undergo dramatic remodeling during epithelial-to-mesenchymal transition (EMT).[6] As EMT begins, these structures undergo disassembly, dismantling cell-cell adhesion while redistributing or degrading junctional proteins. Among these, adherens junctions act as central regulators, coordinating the integrity of the entire junctional network—including tight junctions and desmosomes. Cadherins, the transmembrane proteins within adherens junctions, directly mediate intercellular adhesion.[7] Notably, E-cadherin—a hallmark of healthy epithelial tissues—is frequently lost in metastatic cancers, and experimental suppression of its function transforms epithelial cells into invasive, motile populations.[8] This shift often coincides with upregulated N-cadherin expression, a process termed cadherin switching, which is tightly linked to tumor progression.[7]

The TME is a pathologically altered ecosystem that dynamically evolves during cancer progression, driven by intricate cell-cell signaling networks.[9] Integrating multi-omics data offers a powerful lens to map tumor heterogeneity and decode the complex signaling pathways underlying these interactions.[10] Deciphering the unique molecular pathways driving tumor subtypes enables the discovery of novel therapeutic targets, paving the way for precision therapies tailored to each subtype's molecular profile.[11]

3. Drug-resistant tumor cells communicate directly with immune cells

Cancer cells are not only influencing healthy surrounding tissues with their communication tactics. There is growing evidence that tumors can signal directly to non-cancer cells within the TME. As cancer cells evolve, they acquire resistance to therapy by altering communication with non-malignant cells in the TME (Figure 2). However, the specific interactions between malignant and non-malignant cells that cause this drug resistance remain largely unknown.

Scientists have employed advanced single-cell RNA sequencing to determine the gene expression profile and an immune cell classifier to understand the immune composition of the different tumor biopsy samples.[12]

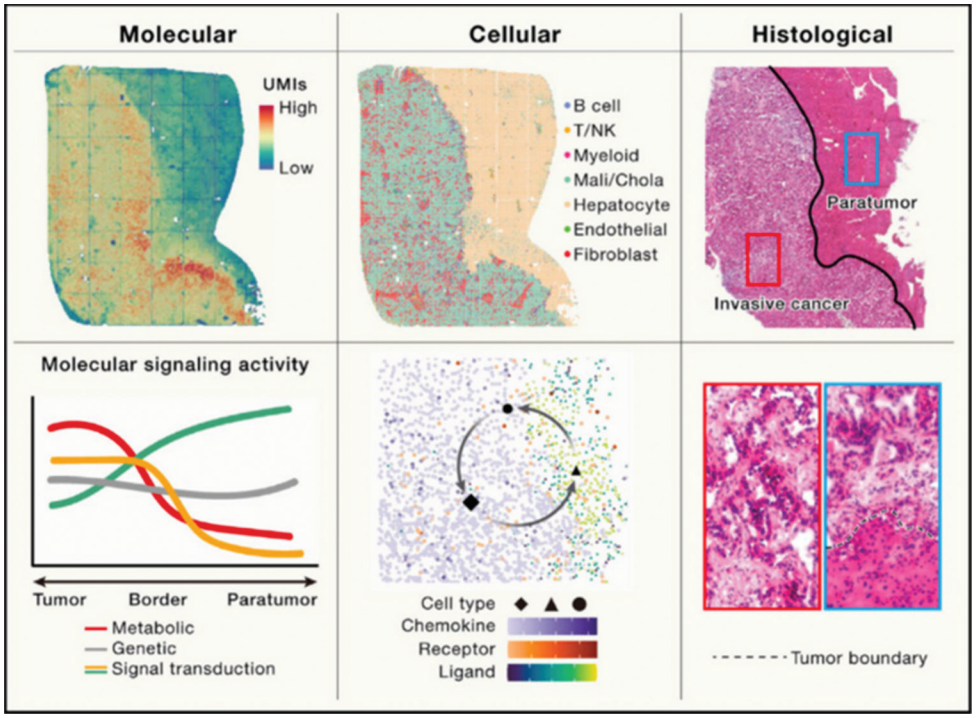

Figure 1. Illustration of how decoding of cell-to-cell communication and signaling can uncover pathogenesis and therapeutic strategies of cancer. The figure was created with BioRender.com.

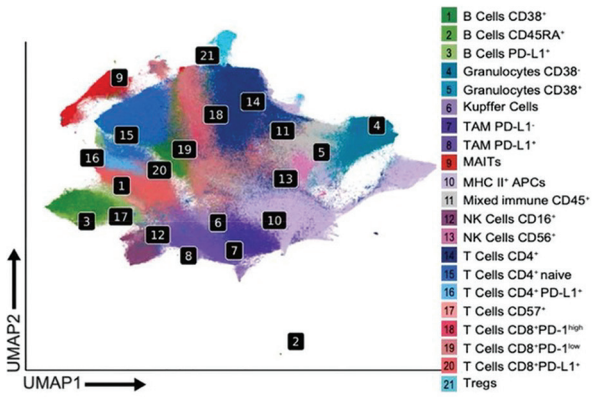

Figure 2. A representative tumor tissue image showing immune cell-type annotation in tumor microenvironment which is segmented to identify individual cells and their associated marker expression profiles. Figure was created using ×10 Genomics Visium Spatial Software Suite.

Their study produced an overall network map of how strongly these cell types are communicating. The results of this network analysis is that the resistant tumor cells lacked normal communication with immune cells but were sending plentiful immunosuppressive signals to macrophages. Cancer cells can alter the phenotypes of macrophages and myeloid cells, and the role of cancer-associated fibroblasts and endothelial cells that can support the growth of the cancer by producing capillary network that provides resources have been described previously.[13] What was unclear before this study was which of these cell types play these roles.

Preclinical research highlights that immune evasion mechanisms emerge early in tumor development. For example, single-cell analysis of premalignant mammary tumors (driven by *BRCA1* and *p53* mutations in a transgenic model) uncovered immunosuppressive microenvironments marked by elevated regulatory T cells (Tregs) and tissue-resident macrophages, even at precancerous stages.[14] Parallel findings in pancreatic cancer models revealed that mutant KRAS-driven lesions progress faster when combined with tissue damage mediated by the pro-inflammatory signal interleukin (IL)-33.[15] Administering IL-33 to mice with KRAS mutations induced epigenetic dysregulation and accelerated pancreatic intraepithelial neoplasia, demonstrating how environmental stressors interact with genetic changes to activate cancer-promoting pathways. These studies underscore that the timing and drivers of immune evasion during tumor initiation vary depending on tissue type, initiating mutations, and host factors.

Chronic inflammation creates a permissive environment for cancer development by hijacking immune cell interactions that suppress anti-tumor defenses. Inflamed tissues often exhibit Th2-polarized immunity

and an influx of immunosuppressive myeloid cells, which release reactive oxygen species, pro-inflammatory cytokines, chemokines, and angiogenic factors. These mediators drive tissue damage, DNA mutations, vascular dysfunction, and matrix remodeling—processes that fuel tumor initiation and progression.[16] Classic examples include inflammatory bowel disease (linked to colorectal cancer), chronic hepatitis or fatty liver disease (associated with hepatocellular carcinoma), and asbestos-induced inflammation (a precursor to mesothelioma). Obesity-related inflammation similarly elevates risk for malignancies such as breast and endometrial cancers.[17]

Whether tumors arise from pre-existing inflammation or trigger inflammation during early growth, most cancers share mechanisms to evade immune surveillance. Progressing tumors frequently exclude or impair anti-tumor immune cells (T cells, natural killer cells, and dendritic cells) while recruiting pro-tumor myeloid populations like macrophages and neutrophils.[18,19] This dual strategy fosters an immunosuppressive, pro-angiogenic niche that supports tumor survival and spread.

4. Long-range cell communication via tumor exosomes

Exosomes are extracellular vesicles ranging from 30 to 150 nm in diameter, released by various cell types, including tumor cells. They can induce apoptosis, modulate the immune system, and serve as biomarkers for diagnosis. As a crucial component of cell-to-cell communication, exosomes regulate the TME and are involved in the development, progression, and metastasis of numerous cancers.[20,21]

Tumor-derived exosomes contain proteins, nucleic acids, lipids, and metabolites that act as effective messengers between tumor cells and various other cells, including immune cells, vascular endothelial cells, and mesenchymal cells (Figure 3). This communication significantly impacts tumor biological activities such as immune regulation, angiogenesis, and EMT, which in turn influence tumor cell growth, proliferation, and metastasis.[22]

Emerging research underscores exosomes as dynamic mediators of intercellular communication, facilitating the transfer of proteins, mRNAs, miRNAs, and oncoproteins to recipient cells.[23] These cargo molecules modulate diverse processes such as immunoregulation, extracellular matrix remodeling, and growth factor signaling, which collectively influence tumor progression.[24] Within the TME—a network of tumor-associated fibroblasts, immune cells, and osteoblasts—exosomes play multiple roles: they enhance tumor cell proliferation, confer chemotherapy resistance, and regulate stromal cell behavior. Stromal cells in the TME internalize exosomal miRNAs, mRNAs, and proteins, which subsequently orchestrate pro-tumorigenic signaling cascades.[25]

Targeting tumor-derived exosomes may positively impact cancer therapy. Exosomes secreted by malignant cells have a tumor-promoting effect, and tumor cells generally secrete more exosomes than normal cells. These secretomes carry mRNA, miRNAs, lncRNAs, and proteins that can serve as biomarkers for cancer diagnosis and prognostic monitoring.[26] In addition, exosomes play a crucial role in therapy resistance. They carry specific payloads that can transfer resistant phenotypes to sensitive cancer cells, conferring resistance to chemotherapy and radiation by altering the cell cycle and inducing anti-apoptotic processes. Exosomes may also inhibit the entry of chemotherapeutic agents into target cancer cells, affecting the efficacy of chemotherapy.[21] Therefore, targeting tumor exosomes may offer a new direction for cancer therapy, and relevant clinical trials are already underway.

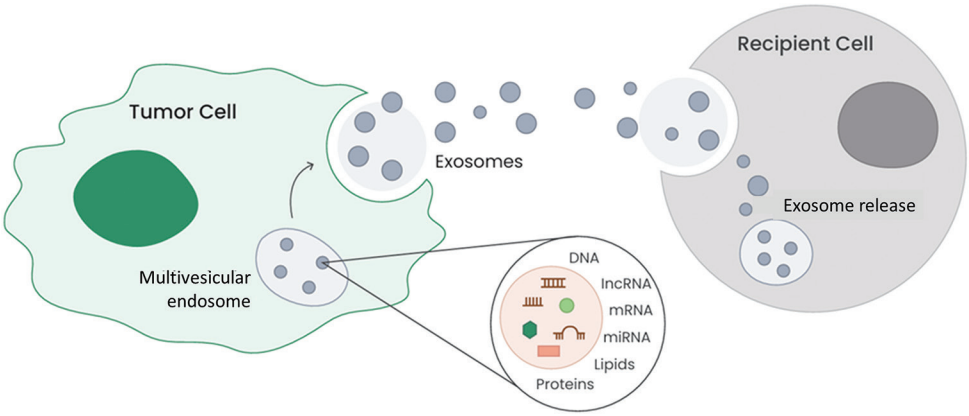

Figure 3. Exosomes mediate cell–cell communication locally and systemically. When reaching the recipient cell, tumor-derived donor exosomes can either trigger signaling by directly interacting with extracellular receptors or be uptaken by direct fusion with the plasma membrane and get internalized. The figure was created with BioRender.com.

4.1. Cell-to-cell communication and metastasis

Metastasis occurs when cancer cells detach from their original site and travel through the bloodstream to establish themselves in other organs, such as the lungs, bones, brain, or liver. Metastasis is the leading cause of death from cancer.[27] The primary tumor rarely causes death; instead, it is the formation of new tumors in distant organs that leads to patient mortality. The process of metastasis is highly inefficient, requiring cancerous cells to undergo numerous changes to survive their journey and thrive in a new environment. Intercellular communication enhances this process in various ways. For instance, tumor cells may collaborate with lymphocytes and/or macrophages to create a pro-tumor environment.[28] Tumor cells sometimes migrate together, resembling an island moving through the bloodstream with its own microenvironment, in search of a new site. These cells alter their communication with non-cancerous cells, such as those in the vasculature or immune system, to ensure their survival. This cell editing process generates signals that favor and support the tumor cells rather than normal cells.[29]

Migrating tumor cells, known as circulating tumor cells (CTCs), have been found to have a significantly higher success rate in forming metastatic tumors when they cluster together—up to 50 times higher.[30] Cell-to-cell communication within the CTC cluster helps these tumor cells survive the harsh environments they encounter during metastasis. In the bloodstream, CTCs face strong physical pressures from flow and constriction, as well as attacks from immune cells. In distant tissues, the growth environment differs greatly from the original tissues, causing many CTCs to die or cease growing permanently. However, CTCs in a cluster gain protection through cell-to-cell communication, enabling them to survive in the bloodstream and successfully regrow in distant organs.[31] Investigators have thereby focused on rapidly testing patient tumor cells for metastatic potential rather than growth. This approach allows scientists to determine within a few hours which drugs can most effectively reduce the risk of metastasis for the specific cells from a patient's tumor.[32]

The electrical activity of cells has been associated with cancer's ability to metastasize. Research has shown that prostate cancer cells can modify their baseline voltage to open sodium ion channels in their membranes. By opening these channels, cancer cells allow ions to enter, increasing their likelihood of metastasizing. Conversely, closing these channels could potentially prevent metastasis.[33] On the other hand, the loss of function of the sodium leak channel non-selective protein (NALCN) in gastrointestinal cancers promotes metastatic disease by increasing the release of CTCs. By considering metastasis as a "normal phenomenon" rather than something unique to cancer, the researchers found that NALCN regulates the dissemination of both normal and cancerous epithelial cells, thereby distinguishing the process of metastasis from cancer.[34] These normal disseminated epithelial-like cells can "metastasize" to distant organs. However, instead of forming tumors, they integrate into normal structures within these tissues, such as kidney tubules. For this process to occur, the seeded circulating epithelial-like stem cells must interact with neighboring cells in their new environment to mimic or adapt to a new cell state.

5. Cell communication research is changing the face of cancer treatment

Research has transformed our understanding of cancer and, consequently, our approach to treatment. We now know that different parts of a tumor communicate to coordinate growth, and blocking this communication could lead to new treatment strategies. Additionally, tumor cells can disguise themselves, tricking the immune system into perceiving them as "normal" cells. They engage with receptors to mask the tumor, adopting the tissue's features to blend in. This cell-to-cell communication allows tumor cells to interact with normal cells, enabling their survival. Significant efforts and technological advancement are underway to disrupt this masking process and reveal the true identity of tumor cells (Figure 4). For instance, immune checkpoint inhibitors are being used to block the masking signals, allowing the immune system to recognize and inhibit tumor cells.[35]

Several types of immunotherapies are effective in treating cancer, including monoclonal antibody therapies designed to recognize specific proteins on cancer cells, mimicking the body's natural response. These therapies hijack cell-to-cell communication, allowing drug treatments to be delivered directly into the tumor to kill the disease. Examples include trastuzumab and rituximab, which work in different ways to either kill cancer cells or stop them from growing.[36] Another approach is chimeric antigen receptor T-cell therapy, a type of adoptive cell transfer. This involves harvesting a patient's immune cells and modifying them to recognize and target tumor cells. A receptor designed to identify a specific protein on the cancer cells is added to the immune cells. Once these modified cells are administered back to the patient, they target and kill the tumor.[37]

Future treatments will continue to focus on blocking cell-to-cell communication, and several existing therapies are already used to treat breast cancer. These include selective estrogen receptor modulators like tamoxifen and

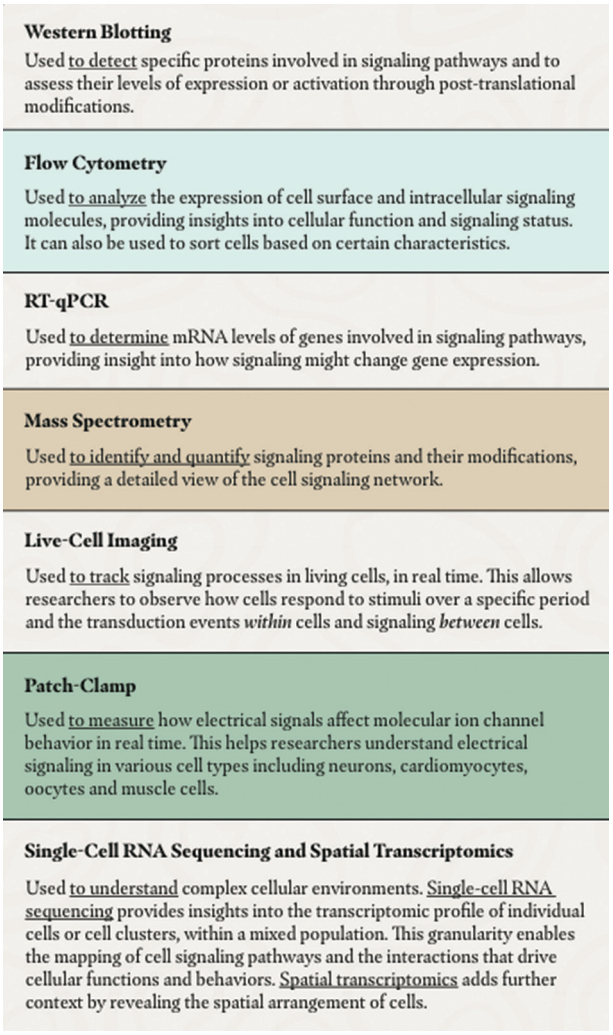

Figure 4. A list of advanced technologies used to analyze cell-to-cell communication and signal transduction in the complex tumor microenvironment. Researchers usually combine multiple technologies to minimize limitations and improve accuracy.

raloxifene, which block the estrogen signals that promote breast cancer cell growth.[38] Additionally, aromatase inhibitors such as letrozole, anastrozole, and exemestane work by blocking estrogen production.[39] Research is increasingly focusing on metastasis due to the scarcity of drugs targeting these metastatic steps and the limited options for closely monitoring the metastasis process in patients.

6. Conclusion

For many years, tumors were seen as masses of rogue cells growing uncontrollably due to abnormal cell signaling pathways. However, as our understanding of the disease deepens, it becomes clear that the communication pathways cancer cells use among themselves and with their microenvironment are far more sophisticated.

Cancer cells engage in complex interactions with neighboring cancerous and stromal cells through direct cell-to-cell contact and paracrine signaling. Long-range communication with distant organ sites is facilitated by extracellular vesicles, exosomes and signaling molecules such as cytokines, hormones, and growth factors. These signaling events are crucial for cancer cell survival, metastasis, and immune evasion. To effectively combat cancer initiation, progression, and metastasis, it is essential to understand how cancer cells communicate within their microenvironment and with distant organ sites.

As cancer evolves and evades treatment, therapies must also adapt. New research, particularly on cell-to-cell communication, is continually enhancing our understanding of how to treat and manage this devastating disease.

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Conflict of interest

The authors declare they have no competing interests.

Author contributions

Conceptualization: Chen Yeh
Writing – original draft: Chen Yeh
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Consent for publication

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ACCSCIENCE PUBLISHING

Tumor Discovery

CASE REPORT

Diffuse large B-cell lymphoma in the splenic hilar lymph node mimicking an intrasplenic lesion

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Abstract

Diffuse large B-cell lymphoma (DLBCL) in the splenic hilar lymph node mimicking an intrasplenic lesion is considered rare in the literature. This case is discussed as a form of a primary splenic DLBCL and as a stage I/II abdominal DLBCL. Primary splenic DLBCL was previously defined as a lymphoma confined to the spleen, with or without involvement of the hilar lymph node or distant lesions. However, the condition is not included in the 5th edition of the World Health Organization classification. In this report, we describe the case of a 63-year-old Japanese male who presented with a 5 cm ^{18}F-fluorodeoxyglucose-avid mass identified on imaging, presumed to be an intrasplenic mass. Subsequent splenectomy confirmed that the mass was DLBCL originating from the splenic hilar lymph node, distinctly separated from the spleen and the tail of the pancreas. Postoperatively, the patient responded well to treatment comprising three courses of a combined regimen of polatuzumab vedotin, rituximab, cyclophosphamide, daunorubicin, and prednisolone. This case underscores the importance of caution when diagnosing intrasplenic lesions based on imaging, as the lesions may be located outside the spleen.

Keywords: Diffuse large B-cell lymphoma; Splenic hilar lymph node; Non-intrasplenic lymphoma; Positron emission tomography-computed tomography

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1. Introduction

Splenic neoplasms encompass various tumors, such as splenic hemangioma, lymphangioma, various subtypes of lymphomas, and angiosarcoma, which must be differentiated for accurate diagnosis and management. In the 5th edition of the World Health Organization classification of splenic B-cell lymphomas and leukemias, diffuse large B-cell lymphoma (DLBCL) is not listed as a specific subtype.[1] However, although rare, primary splenic DLBCL has been documented in several case reports and case series[2-13] (Table 1). According to Shimizu-Kohno *et al*.,[2] an analysis of 115 specimens of splenic lymphoid neoplasms in Japan revealed that DLBCL was the most common

Table 1. Subtypes of non-Hodgkin lymphomas in the spleen

Subtypes	References
B-cell type	
[a]Splenic diffuse red pulp small B-cell lymphoma	1
[a]Splenic marginal zone lymphoma	1
[a]Splenic B-cell lymphoma/leukemia with prominent nucleoli	1
[a]Hairy cell leukemia	1
Primary splenic diffuse large B-cell lymphoma	2-10
Fibrin-associated large B-cell lymphomas in splenic cysts	11
Primary splenic CD10-positive small B-cell lymphoma/ follicular lymphoma.	12
T-cell and other cell type	
[a]Hepatosplenic T-cell lymphoma	1
Splenic T/NK cell lymphoma	2
Splenic micronodular T-cell/histiocyte-rich large B-cell lymphoma	13

Note: [a]Refers to the World Health Organization Classification of Tumors, 5th edition (Hematolymphoid tumors-Part B).[1] Other splenic lymphomas are based on literature surveys.
Abbreviation: NK: Natural killer.

type (46 cases), followed by splenic marginal zone lymphoma (28 cases), and follicular lymphoma (11 cases).[2] Shimono *et al.*[3] classified primary splenic DLBCL into two categories: (i) type A, which includes cases with or without lymphadenopathy of splenic hilum, and (ii) type B, characterized by cases with involvement of the bone marrow, liver, or peripheral blood, in addition to the splenic lesions.[3] Here, we report a rare case in which an intrasplenic mass was initially suspected based on imaging studies but was later identified as DLBCL in the splenic hilar lymph node without intrasplenic lesions after splenectomy examination.

2. Case presentation

A 63-year-old Japanese man (174 cm, 64 kg), a hepatitis B virus carrier, presented with a hypoechoic spleen mass discovered during a routine annual abdominal ultrasound. Notably, he reported no symptoms of left upper abdominal pain or splenomegaly. His laboratory findings included a white blood cell count of 5,100 cells/μL, hemoglobin at 14.5 g/dL, platelet count of 250,000 platelets/μL, alanine aminotransferase at 14 U/L, lactate dehydrogenase at 209 U/L (reference range: 124 – 222), total bilirubin of 0.58 mg/dL, total protein of 7.3 g/dL, and albumin at 4.5 g/dL. Serological markers indicated active hepatitis B infection (HBsAg positive, HBsAb negative, HBcAb positive, HBeAb positive, and HCVAb negative). In addition, serum C-reactive protein was 0.1 mg/dL, with immunoglobulin G and immunoglobulin M levels recorded at 1,140 mg/dL and 47 mg/dL, respectively. Tumor marker studies showed a slightly high soluble interleukin-2 receptor (666 U/mL, reference range: 122 – 496), whereas other tumor markers (CA19-9 and CEA, determined by chemiluminescent enzyme immunoassay, DUPAN-2, determined by enzyme immunoassay, and SPAN-1, determined by radioimmunoassay) were within normal limits.

Contrast-enhanced computed tomography (CT) revealed a mass measuring 5 cm × 4 cm with indistinct margins in the middle of the spleen (Figure 1A). ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET)-CT confirmed the presence of an FDG-positive mass measuring 5 cm with a maximum standardized uptake value of 28.3 (Figure 1B). These imaging studies suggested an intrasplenic lymphoma.

The spleen (where the mass was initially thought to be located) and the tail of the pancreas were resected *en bloc* through robot-assisted laparoscopic surgery, along with dissection of regional small lymph nodes. Gross examination revealed a white, well-defined 5 cm soft mass between the spleen and pancreas (Figure 1C).

Formalin-fixed paraffin-embedded tissue specimens were stained with hematoxylin-eosin and specific antibodies, including CD3 (Roche Diagnostics, United States), CD5 (Roche Diagnostics, United States), CD10 (Nichirei Biosciences, Japan), CD20 (Roche Diagnostics, United States), CD23 (Nichirei Biosciences, Japan), BCL-2 (Roche Diagnostics, United States), BCL-6 (Roche Diagnostics, United States), Ki-67 (Roche Diagnostics, United States), and MUM-1 (Roche Diagnostics, United States), for immunohistochemistry (IHC). IHC was considered positive if more than 30% of tumor cells were positively stained. Ki-67 labeling index was estimated on the "hot spot" by simple visual inspection ("eyeballing").

Microscopic examination of the resected mass revealed that it did not invade the splenic parenchyma or pancreatic tissue, and no lymphoma cells were found in the regional lymph nodes. Histopathological analysis confirmed the diagnosis of DLBCL, specifically of the germinal center B-cell type (Figure 1D-F). The bone marrow was free from lymphoma cell involvement, leading to a diagnosis of stage I DLBCL originating from the splenic hilar lymph node. The karyotype was not assessed in this case. Fluorescence *in situ* hybridization (Vysis™ LSI® IGH/MYC, CEP 8 Tri-color, Dual Fusion Translocation Probe, Abbott Molecular Inc., United States) analysis was performed on the lymphoma cells from the DLBCL tissue. Results indicated negative for the *IGH-MYC* translocation, t(8;14)(q24;q32).

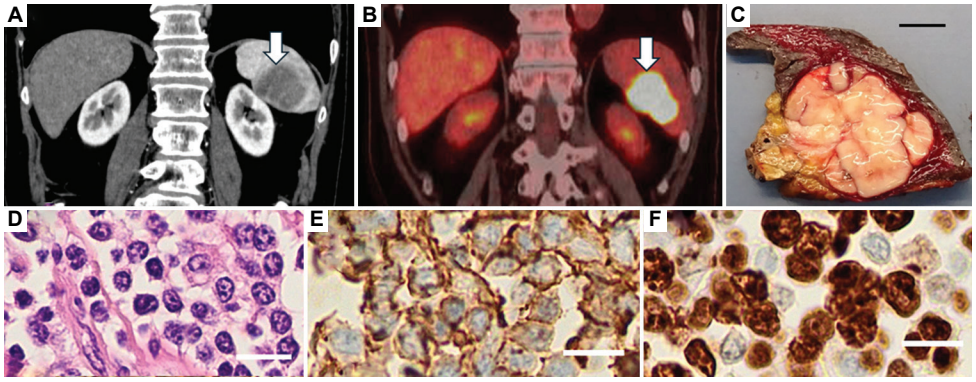

Figure 1. Enhanced computed tomography shows a 4 cm × 3 cm mass (white arrow) in the middle of the spleen. (A) The margin of the mass was indistinct. (B) Positron emission tomography-computed tomography demonstrates an ^{18}F-fluorodeoxyglucose-avid mass in the same area (arrow). (C) A white, well-defined 5 cm soft mass (shown as a dissected surface) is located outside the spleen, magnification: ×4, scale bar: 1 cm. Histology of the mass derived from the splenic hilar lymph node revealed diffuse large B-cell lymphoma as described in the text: (D) Hematoxylin & Eosin stain, (E) CD20 stain, and (F) Ki-67, magnification: 400×, scale bar: 50 μm. Positive stains for CD10, BCL-2, and BCL-6 are not shown.

Postoperatively, the patient experienced minor complications related to pancreatic leakage during the first 2 months but did not encounter any serious side effects, except for grade 1/2 cytopenia following three cycles of polatuzumab vedotin, rituximab, cyclophosphamide, doxorubicin, and prednisolone combination therapy (PvRCHP regimen).[14] As of 5 months post-diagnosis, the patient has been doing well without any recurrence of lymphoma.

3. Discussion

This case was initially suspected to involve a primary splenic mass based on imaging studies that mimicked an intrasplenic lesion (Figure 1A and B). As listed in Table 1, several B-cell and other lineage lymphomas must be differentiated if an intrasplenic lymphoma is considered. However, in this case, gross and microscopic examinations of the formalin-fixed specimen revealed that the lymphoma originated from the splenic hilar lymph node rather than from within the spleen. The enhanced CT showed a mass with an indistinct margin (Figure 1A), which did not resemble typical imaging of a splenic cyst found in the fibrin-associated large B-cell lymphoma, a rare subtype of Epstein-Barr virus-associated lymphoma and distinct from typical DLBCL.[11,15] In retrospect, it was hypothesized that the CT appearance was due to external compression of the spleen by the mass.

In our search for literature on DLBCL localized to the splenic hilar lymph node, similar to our case, we found no relevant reports in PubMed or Google Scholar. This absence raises a critical question regarding the classification of our DLBCL: Is it classifiable as a primary splenic DLBCL-related lymphoma or as one of the abdominal nodular DLBCLs? Shimono *et al.*[3] conducted a comparative study of primary splenic DLBCL (66 cases) and control DLBCL (309 cases). They noted that patients with primary splenic DLBCL exhibited specific clinical characteristics, including a higher hepatitis C virus antibody prevalence, the presence of B symptoms, poor performance status, and CD5 positivity. Notably, the response to initial treatment was better in the primary splenic DLBCL group ($P < 0.05$), whereas the control group required radiation therapy.[3] However, our case did not exhibit these characteristics associated with primary splenic DLBCL.

The outcomes of primary splenic DLBCL demonstrate varying treatment responses and survival rates across different populations. In Japan, a study reported that among 66 patients, 40 patients received R-CHOP therapy, with 39 achieving complete remission.[3] Conversely, in Israel, among 87 patients, splenectomy was carried out in 39 patients, with the majority receiving chemotherapy. At a 5-year follow-up, the overall survival rate was observed to be 77%, whereas the disease-free survival rate was 67%.[16] In addition, Yonghao *et al.*[7] conducted an analysis involving 347 patients with primary splenic DLBCL. They found that combined treatment with splenectomy and chemotherapy demonstrated better outcomes compared to other treatment modalities, such as no treatment, splenectomy alone, or chemotherapy alone.

In contrast, regarding stage I/II DLBCL treatment outcomes, Persky *et al.*[17] reported on 132 patients with non-bulky (<10 cm) stage I/II DLBCL, who received three cycles of R-CHOP (with an additional cycle for cases showing negative PET/CT). In this cohort, only six patients experienced disease progression, and three succumbed to lymphoma, with a median follow-up of 4.92 years (range: 1.1 – 7.7 years).[17] Although previous chemotherapy utilized four cycles of R-CHOP on primary splenic DLBCL

and stage I/II DLCBL,[3,4,6,8,10,17] our case was managed postoperatively with three courses of the PvRCHP regimen due to concerns regarding potential poor prognosis.

4. Conclusion

In this case, after surgical resection and subsequent gross and microscopic examinations, it was determined that the mass originated from the splenic hilar lymph node, despite pre-operative imaging studies (CT, PET/CT) suggesting an intrasplenic tumor. Thus, even when imaging studies indicate an intrasplenic mass, caution must be exercised regarding the possibility of lymphoma located outside the spleen, potentially within the splenic hilar lymph node.

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Conflict of interest

The authors declare no conflicts of interest.

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Ethics approval and consent to participate

The work was carried out following the Declaration of Helsinki as revised in 2013. This case report was approved by the institutional review board (Uji-Tokushukai Medical Center Ethics Committee; IRB approval No. 2024-45). The patient provided written consent to participate in this study.

Consent for publication

Written informed consent was obtained from the patient.

Availability of data

Data are available upon request from the authors.

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ACCSCIENCE PUBLISHING

Tumor Discovery

CASE REPORT

Desmoplastic small round cell tumor: A case report

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Abstract

Desmoplastic small round cell tumors are extremely rare, occurring primarily in young males. This disease is characterized by the reciprocal translocation of the *EWS-WT1* fusion gene. Treatment for this tumor is multidisciplinary, including surgery, chemotherapy, and radiation therapy. The disease progresses rapidly and even with complete resection, local recurrence, and distant metastasis is likely to occur and the prognosis is poor. This report describes a case of primary ovarian cancer in a 33-year-old woman. The pre-operative test showed elevated CA125, CA19-9 and carcinoembryonic antigen, and hypercalcemia. Staging laparotomy was performed without residual tumor. The clinical stage was IIB, and adjuvant chemotherapy was performed. Immediately after the end of chemotherapy, multiple lymph node metastases, and the patient subsequently experienced repeated recurrences and died 10 months after surgery. In the future, it is desirable to establish standard treatment for desmoplastic small round cell tumors by analyzing more cases.

Keywords: Case report; Desmoplastic small round cell tumor; Ovarian cancer; Chemotherapy; Multikinase inhibitor; Immune checkpoint inhibitor

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1. Introduction

Desmoplastic small round cell tumor (DSRCT) is an extremely rare malignant tumor. DSRCT is more prevalent in men than in women, with a male-to-female ratio of 4:1. DSRCT rarely occurs in the ovaries; it mainly occurs in adolescents and young adults, and tends to cause peritoneal dissemination, lymph-node metastasis, and distant metastasis to the liver and lungs. A molecular biological feature of DSRCT is the reciprocal translocation of the *EWS-WT1* fusion gene. Treatment for DSRCT includes multidisciplinary treatments such as surgery, chemotherapy, and radiation therapy, and may include radiofrequency ablation, gamma knife, cryoablation, and vascular embolization in cases of recurrence. In the present case, despite complete resection, lymph node and distant organ metastases occurred early. Recently, molecular-targeted drug treatment has also been expected, and there have been reports of long-term growth inhibitory effects of the multikinase inhibitor sunitinib.[1] However, in the case reported herein, no response was obtained despite treatment with a protocol that included lenvatinib.

2. Case presentation

Here, we report the case of a 33-year-old woman with three pregnancies and three births. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

The patient gave birth in November 2020, and no abnormalities were found during pregnancy or post-partum examination. In October 2021, the patient experienced frequent lower abdominal pain. She visited a nearby obstetrics and gynecology clinic. Ultrasonography revealed a 10 cm solid pelvic tumor. Therefore, she was referred to our hospital with suspected ovarian cancer.

The examinations conducted at our hospital, including computed tomography (CT) (Figure 1) and magnetic resonance imaging (MRI) (Figure 2), revealed a large ovarian tumor measuring approximately 12 × 10 × 10 cm, with no evident lymph node or distant metastases. Blood test results were as follows: No anemia – hemoglobin, 12.1 g/dL; cancer antigen 125 (CA125), 119.2 IU/mL; cancer antigen 19-9 (CA19-9), 163 IU/mL; carcinoembryonic antigen (CEA), 7.8 ng/mL; neuron-specific enolase (NSE), 10.0 ng/mL; and alpha-fetoprotein <2.0 ng/mL. Although she was asymptomatic, her serum calcium level was significantly high (15.0 mg/dL). She was hospitalized on October 8, 2021, for ovarian cancer treatment, and elcatonin was pre-operatively administered.

The operation was performed on October 11. Upon laparotomy, a solid tumor of approximately 10 cm in the left ovary was found (Figure 3), without any ascites or minor adhesions between the tumor surface and the rectum. No significant lymph node swelling was observed. Rapid intraoperative histopathological examination indicated malignancy; however, the specific type was indeterminate. We performed abdominal total hysterectomy, bilateral salpingo-oophorectomy, partial omentectomy, and pelvic and para-aortic lymph node dissection. The surgery lasted for 4 h and 14 min, with a total blood loss of 891 mL.

The degree of surgical completion was satisfactory. The patient's post-operative course was uneventful, and she was discharged from the hospital on October 18 without any perioperative complications. In addition, histopathological examination revealed highly atypical small round cells with swollen nuclei and a high nucleus-to-cytoplasm ratio, which proliferated densely and clustered to form alveolar nests. Various degrees of hyaline stroma were also observed between alveolar nests (Figure 4A and B). Immunostaining was positive for WT-1 (Figure 4C), CD99 (Figure 4D), CD56 (Figure 4E), and EMA (Figure 4F). Based on these results, the histopathological diagnosis was DSRCT, clinical stage IIB, and pT2bN0M0.

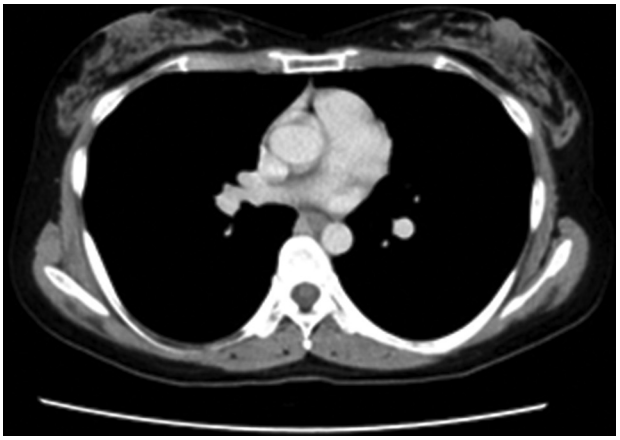

Figure 1. Computed tomography result (contrast computed tomography)

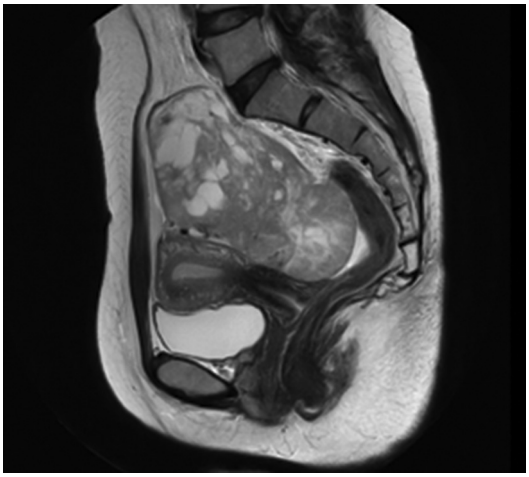

Figure 2. Magnetic resonance imaging result (T2)

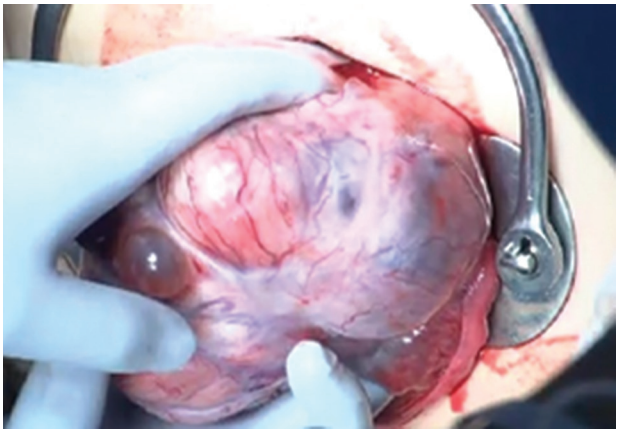

Figure 3. The tumor intraoperatively

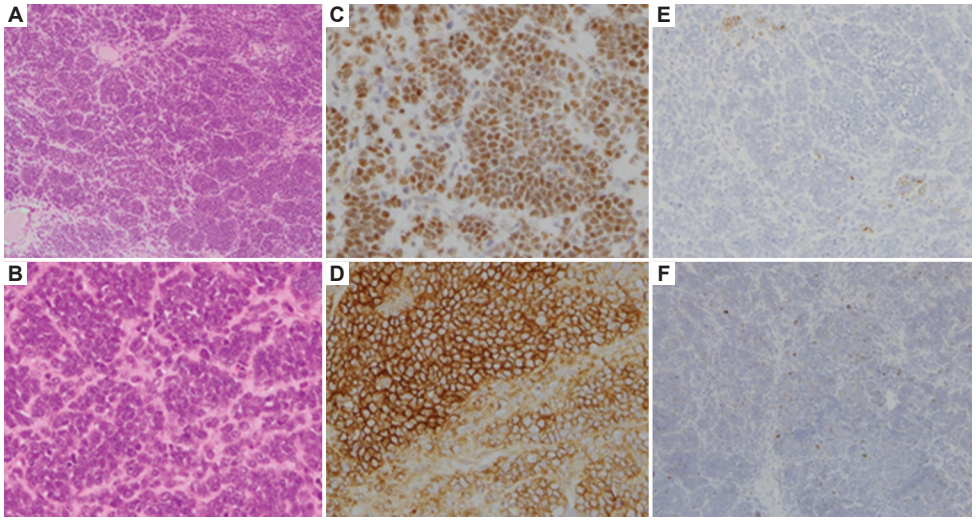

Figure 4. Histopathological findings. (A and B) Hematoxylin & eosin (HE) staining; magnification: ×100 (a), ×400 (b). (C-F) Immunohistochemistry staining for WT-1 (c; ×400), CD99 (d; ×200), CD56 (e; ×200) and EMA (f; ×100).

Consequently, six cycles of paclitaxel, carboplatin, and bevacizumab were initiated on October 27 as adjuvant chemotherapy. During this period, the tumor marker levels did not increase, and a CT scan performed on December 1 showed no evidence of recurrence. The patient continued outpatient follow-ups; however, positron emission tomography (PET) on March 11, 2022, revealed multiple lymph-node metastases in the mediastinum (Figure 5A), para-aortic lymph nodes (Figure 5B), and right obturator nodes (Figure 5C).

Nonetheless, the tumor marker levels were negative. Therefore, radiation therapy (right obturator node, para-aortic lymph node, and mediastinum; total dose: 54 Gy) was administered between March 23 and May 11. Subsequently, CT scans taken on May 17 (Figure 6) revealed multiple liver metastases and left subclavian lymph node metastasis; thus, we started administering lenvatinib + pembrolizumab as second-line chemotherapy on June 1, with informed consent.

A CT scan on July 27, 8 weeks post-treatment, revealed increased liver metastasis (Figure 7); hence, docetaxel + gemcitabine were initiated as third-line chemotherapy on August 3. On August 14, the patient presented to our emergency department with difficulty in breathing and coughing. Upon admission, she was in shock, with a blood pressure of 70/45 mmHg, requiring oxygen at 7 L/min and SatO2 (arterial blood oxygen saturation) at 70%, and exhibited mild consciousness disturbance. Blood tests indicated a hemoglobin level of 7.1 g/dL, platelet count of 18,000/μL, and C-reactive protein level of 33 mg/mL. Subsequent CT images (Figure 8) showed accelerated progression of the liver and multiple lung metastases, with

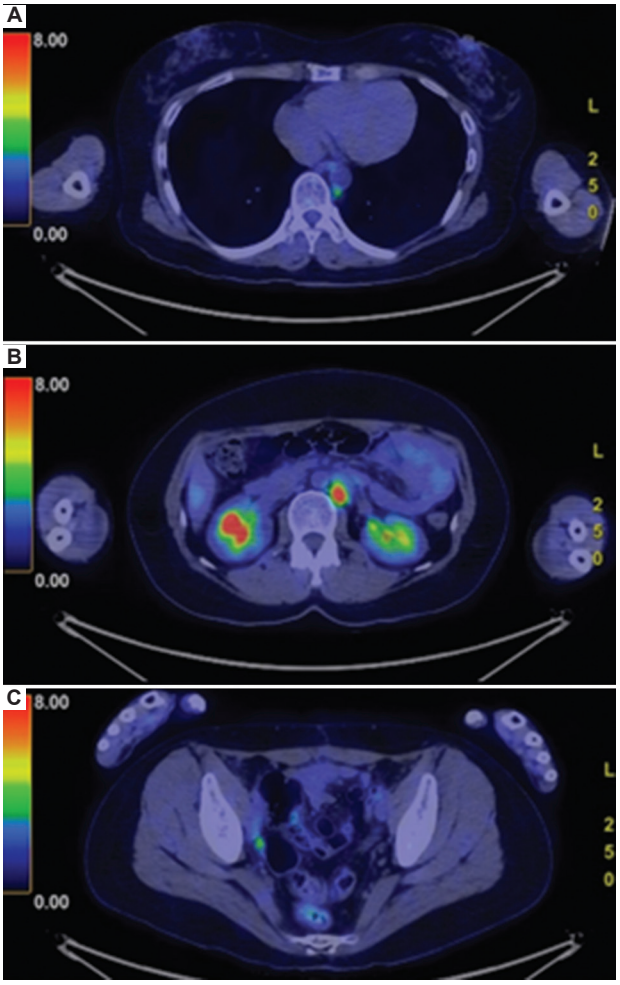

Figure 5. Positron emission tomography. (A) Mediastinal lymph node metastasis. (B) Para-aortic lymph node metastasis. (C) Right obturator lymph node metastasis.

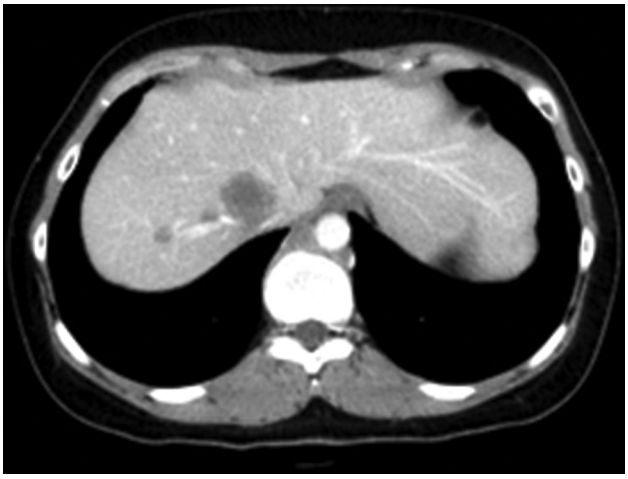

Figure 6. Multiple liver metastases after irradiation (contrast computed tomography)

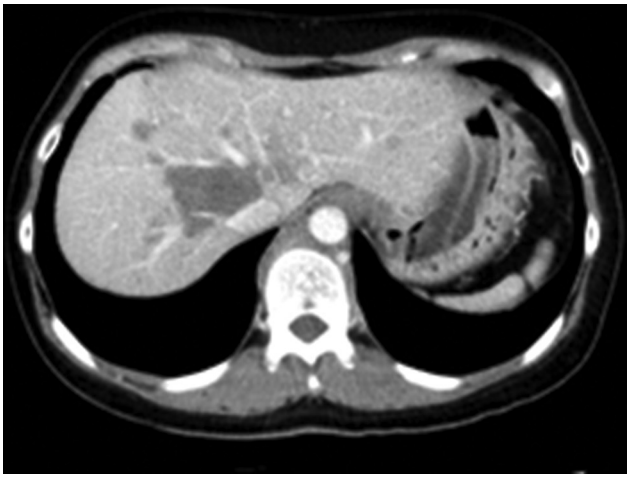

Figure 7. Increased liver metastasis after administration of lenvatinib and pembrolizumab (contrast computed tomography)

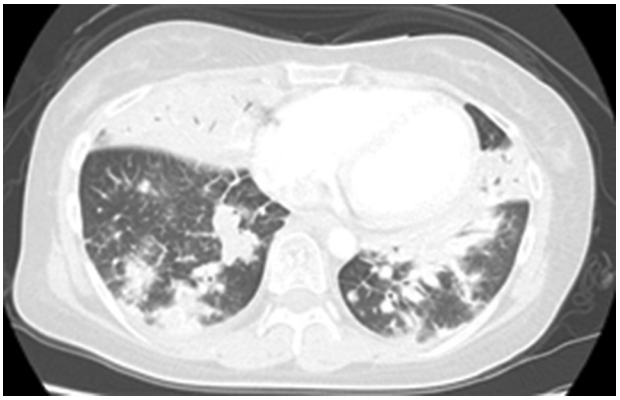

Figure 8. Computed tomography scan of the patient before death

no signs of pulmonary embolism. Her condition quickly worsened, leading to cardiopulmonary arrest, and she died 3 h after admission. No autopsy was conducted according to the wishes of the patient's family.

3. Discussion

DSRCTs were first reported by Gerald and Rosai in 1989.[2] This condition occurs most frequently in young people, and its peak incidence has been reported in people in their 30s. It predominantly affects men and is exceedingly rare in women. While it most frequently presents in the peritoneum and omentum, occurrences at other sites, including the pleura, ethmoid sinus, scalp, hands, posterior cranial fossa, pancreas, ovaries, paratesticular region, and kidneys, have been documented.[3-5]

Clinical symptoms of primary gynecological disease are abdominal distension with abdominal pain and ascites, mass palpation, constipation, anorexia, and weight loss.[6,7] More than 40% of patients have distant metastases at the time of the initial diagnosis, mostly to the liver, lungs, bones, and lymph nodes. Most cases develop as intra-abdominal masses, which have an average size of 11 cm by the time that they are diagnosed.[3] Although diagnostic imaging is useful, it often shows non-specific findings.[8] The patient had previously presented with asymptomatic lower abdominal pain. The patient was diagnosed as having asymptomatic hypercalcemia; post-surgery, their serum calcium levels decreased to 9.3 – 9.9 mg/dL, and during the period from recurrence to death, it stabilized at 9.1 – 9.7 mg/dL without an upward trend.

Examination findings are often recognized as large tumors with internal heterogeneity on CT scans. Moreover, MRI often shows high signal intensity on T2-weighted images and equal signal intensity on T1-weighted images.[8] PET is often used to accurately detect early post-treatment recurrence.[9] In this case, the imaging was characteristic. The levels of the tumor marker CA125 are known to increase.[10] The tumor measured 10 cm, and pre-operative CT revealed no distant metastasis. In addition, the tumor markers CA125, CA19-9, and CEA values were elevated, NSE remained below the cut-off, and LDH levels were within normal ranges. However, post-recurrence, these markers did not increase, implying that they were not reliable indicators of disease status in later stages. This may reflect a variation in tumor cell populations between the initial treatment and recurrence.

According to the World Health Organization's 2020 classification, the condition is classified as a peritoneal tumor and is characterized by gene translocation, including that of the *EWSR-WT1* fusion gene.[11] Macroscopically, it appears as a solid mass, and the cut surface is white and solid, which is sometimes accompanied by necrosis. Histological features include epithelial-like tumor cells

that exhibit large and small solid alveoli with high connectivity, cord-like structures, tubular structures, and rosette-like arrangements, which proliferate in an invasive manner and are accompanied by desmoplastic stroma.[11] Immunohistologically, the tumor was positive for cytokeratin and was stained with desmin in dots around the nucleus.[11]

These tumors are believed to originate from undifferentiated, multipotent cells. During chemotherapy, tumor cells differentiate into rhabdomyoblast-like cells and express the muscle lineage markers Myo-D and myogenin.[12]

The molecular biological feature of DSRCTs is the reciprocal translocation of the *EWS-WT1* fusion gene. *EWS* is located on chromosome 22q12 and encodes a member of the TET family of RNA-binding proteins. Furthermore, the TET family proteins are thought to be involved in transcription and splicing. The *EWS* gene is also related to other sarcomas, such as *EWS-FLI1* and *EWS-ERG* fusion genes in Ewing's sarcoma. Furthermore, *WT1* is located on 11p13, and the WT1 protein contains a zinc finger DNA-binding region that is responsible for the transcription and post-transcriptional regulation of many target genes. The reciprocal translocation t (22:11) (q12: p13) fuses the N-terminal region of WS with the C-terminal DNA-binding region of WT1. The resulting chimeric protein acts as an abnormal transcription factor that causes desmoplastic small-cell tumors. In addition, the EWS-WT1 fusion protein is thought to regulate the expression of various genes involved in carcinogenesis, such as *IGF-1* receptor, *PDGFα*, *PAX2-2*, *WT-1*, *ENT4*, *TALLA-1*, and *IL2/15Rβ*.[13-16] Although atypical, this mechanism may also be involved in the development of Wilms tumor, hypercalcemic small cell carcinoma of the ovary, gonadal cell tumor, and undifferentiated small round cell tumor, in addition to DSRCT.

Primary treatments include surgery, radiation therapy, chemotherapy, and molecular-targeted drugs. However, no standard treatment exists. Techniques, such as radiofrequency ablation, gamma knife, cryoablation, and vascular embolization, are used to treat metastatic lesions. Surgical therapy, often entailing omentectomy, splenectomy, or lymph node dissection, is commonly performed. Achieving negative margins is typically impractical because of the tumor invasiveness. In this case, although the lymph node and liver metastases recurred, there was no intraperitoneal recurrence, such as peritoneal dissemination or ascites. Despite slight rectal adhesions, no visible residual disease or local pelvic recurrence was detected during surgery, suggesting completeness of the procedure.

Chemotherapy is often performed using the P6 protocol that combines cyclophosphamide, doxorubicin, vincristine, ifosfamide, and etoposide. Other drugs included cisplatin, carboplatin, topotecan, temozolomide, vinorelbine, and irinotecan. High-dose chemotherapy and autologous hematopoietic stem cell transplantation are sometimes performed, but these have not been shown to improve the long-term prognosis.[17] Although DSRCTs are chemotherapy-sensitive, they are prone to local recurrence and chemotherapy alone is not effective enough to cure them. This potentially reflects the diversity of tumor cells. Furthermore, rapamycin, an mTOR inhibitor, reduced EWS-WT1 expression and triggered apoptosis in DSRCT cell lines. However, no clinical benefit has been reported in patients with DSRCTs treated with rapamycin alone.[18] In addition, the tumor progression was reported to be suppressed for 56 weeks when sunitinib, a multikinase inhibitor that targets *VEGFR1*, *2*, *3*, *PDGFR-α*, *PDGFR-β*, *KIT*, *FLT-3*, *RET*, and *CSF-1*, was administered.[1,19] In this instance, due to the delay between surgery and pathological diagnosis, we initiated adjuvant chemotherapy with paclitaxel, carboplatin, and bevacizumab with no evidence of recurrence during treatment, suggesting potential efficacy. After recurrence, lenvatinib plus pembrolizumab was administered because lenvatinib has effects comparable to those of sunitinib; however, the patient showed no response. In addition, the effects of immune checkpoint inhibitors are uncertain.

A report summarizing the treatment results of 66 cases of DSRCTs from the Memorial Sloan Kettering Cancer Center found that while there were no survivors in cases without surgery, the 3-year survival rate of patients who underwent tumor resection was 58%.[20] However, in this case, distant metastasis occurred relatively early, despite a complete surgical procedure, and although it appeared to be localized within the peritoneal cavity, micro-distant metastasis may have already occurred. Thus, the present case appears to highlight a case-specific discrepancy regarding the effect of complete surgical resection on prognosis. Furthermore, if possible, a more precise diagnosis using PET is desirable when formulating a treatment strategy. However, prediction of this disease is impossible, and may not be realistic.

4. Conclusion

Herein, we report a case of primary desmoplastic small round cell ovarian tumor. We hope that, by accumulating such cases, standard treatment can be established.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

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Investigation: Masaru Kanasugi
Writing – original draft: Masaru Kanasugi
Writing – review & editing: Tsuyoshi Honda, Shigeyuki Asano

Ethics approval and consent to participate

The patient and her family were given an oral explanation and written consent was obtained.

Consent for publication

The patient and her family gave consent to publish their data.

Availability of data

Not applicable.

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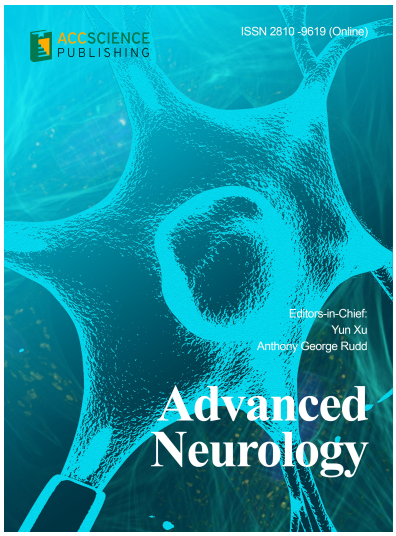

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