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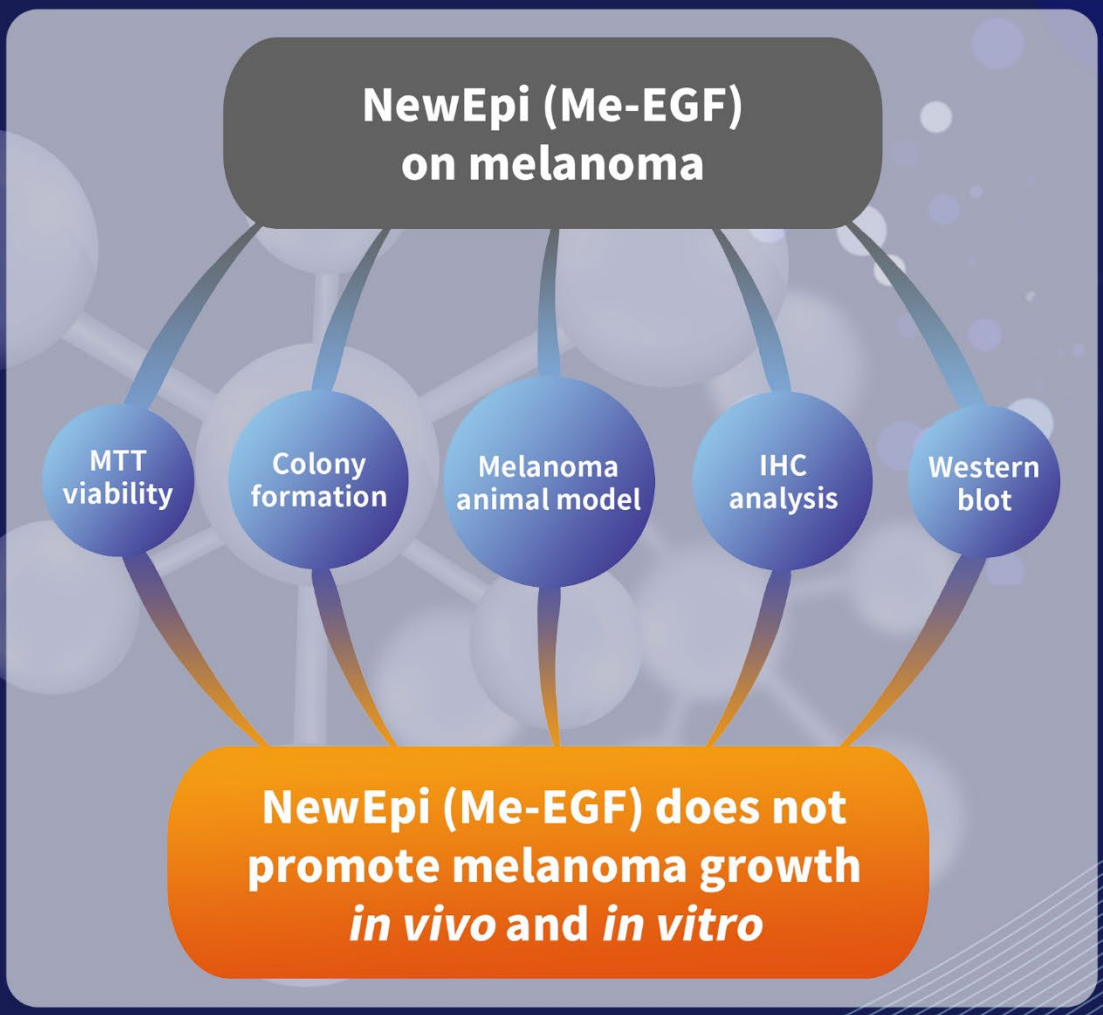

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A graphic illustration of double-stranded DNA

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CONTENTS

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Gene & Protein in Disease

EDITORIAL

Exploring the promise of glycomedicine: A sweet revolution in health care

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Glycomedicine, a burgeoning field at the intersection of glycobiology and medicine, is emerging as a game changer in the health-care realm[1]. While proteins and nucleic acids have historically dominated scientific discourse, the intricate world of carbohydrates is now taking center stage as researchers unravel the profound impact of glycans on human health. This editorial delves into the promising landscape of glycomedicine, shedding light on its potential to revolutionize diagnostics, therapeutics, and preventive medicine[1,2].

Glycans, complex sugar molecules, play pivotal roles in a myriad of biological processes. Depending on how glycans bind to proteins, glycosylation is categorized into N-glycans, O-glycans, C-linked, glypiation, and phosphoglycosylation[1]. From cell signaling and immune response modulation to protein folding and pathogen recognition, the versatility of glycans is staggering. Unlike the relatively straightforward world of genomics and proteomics, deciphering the "glycome" poses a formidable challenge, given the structural diversity and dynamic nature of these sugar chains[1,3].

The unique glycan signatures associated with various diseases offer a treasure trove of diagnostic opportunities[1]. Glycoproteins, adorned with specific sugar structures, undergo alterations in various pathological conditions[1,2]. Detecting these changes can serve as highly sensitive and specific diagnostic markers, potentially facilitating early disease detection and personalized medicine approaches[1,4].

Glycans are increasingly being recognized as both therapeutic targets and agents themselves. Glycan-based therapies hold promise in treating a diverse range of diseases, encompassing congenital disorders of glycosylation, cancer, infectious diseases, and autoimmune disorders[1,4,5]. The manipulation of glycan interactions by researchers is giving rise to innovative approaches for modulating immune responses, impeding pathogen invasion, and precisely targeting specific cancer cells[1-4,6].

The diversity of glycan structures among individuals underscores the importance of glycomedicine. Profound insights into an individual's glycome profile could pave the way for tailor-made therapies that account for variations in disease susceptibility, treatment response, and adverse reactions. Glycomedicine holds the promise of transitioning the prevailing paradigm from a generalized, one-size-fits-all approach to a more precise and effective model of health care[4].

While the potential of glycomedicine is extensive, challenges persist. Theoretically, one might inquire about the existence of a "*paracentral dogma*" that could provide insights into these revolutionary discoveries by considering sugars as the third life codes, following nucleic acids and proteins – the first and second life codes for cellular

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materiality[1,6]. Technically, the analysis of the glycome is demanding and necessitates the utilization of sophisticated tools and techniques, such as mass spectrometry (MS), liquid chromatography (LC), capillary electrophoresis (CE), nuclear magnetic resonance, microarray, cryo-electron microscopy, and glycan imaging[1,2,5]. Each of these methods has its own drawbacks, leading to the application of complementary techniques for a comprehensive analysis of compounds, including gas chromatography-MS, LC-MS, and CE-MS[4,5]. Simultaneously, innovative technologies, automated workflows, and dedicated software are under development[4,5]. Furthermore, the standardization of glycan analysis methods and the establishment of large-scale glycan databases are critical steps for advancing the field. In addition, ethical considerations surrounding glycan-based diagnostics and therapies must be addressed to ensure responsible and equitable use.

Glycomedicine represents a paradigm shift in our understanding of health and disease. The intricate language of glycans, once a mysterious code, is currently in the process of deciphering to unlock novel diagnostic and therapeutic avenues[1,2,4,6]. As research in this field gains momentum, the promise of glycomedicine is poised to revolutionize health care, offering a sweet revolution that could redefine our approach to precision medicine in the not-so-distant future. Navigating this exciting frontier underscores the paramount importance of collaboration among scientists, clinicians, and ethical stakeholders to ensure that the benefits of glycomedicine are harnessed responsibly for the betterment of global health[1,2,4,5].

Conflict of interest

The author declares no conflict of interest.

References

1. Wang W, 2023, Glycomedicine: The current state of the art. *Engineering*, 26(7): 12–15.

   https://doi.org/10.1016/j.eng.2022.03.009

2. Wang W, Yang B, 2023, The glycome and glycomedicine. *Engineering*, 26: 1–2.

   https://doi.org/10.1016/j.eng.2023.05.007

3. Wang W, Jia JT, 1987, Phenotyping of phosphoglucomutase (PGM1) subtypes in Chinese, with special reference to new variant PGM1 China. *Med Sci Law*, 27: 280–282.

   https://doi.org/10.1177/002580248702700408

4. Wang W, 2023, Editorial: Orthodox vs paradox: The roles of glycomics, genetics and beyond in immunity, immune disorders and glycomedicine. *Front Immunol*, 14: 1305552.

   https://doi.org/10.3389/fimmu.2023.1305552

5. Wang Y, Adua E, Russell A, *et al*., 2016, Glycomics and its application potential in precision medicine in China. *Science/AAAS*, 354(6316): 36–39.

   https://doi.org/10.1126/science.354.6319.1601-b

6. Ozdemir V, Arga KY, Aziz RK, *et al*., 2020, Digging deeper into precision/personalized medicine: Cracking the sugar code, the third alphabet of life, and sociomateriality of the cell. *OMICS*, 24(2): 62–80.

   https://doi.org/10.1089/omi.2019.0220

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REVIEW ARTICLE

Unleashing the potential of stem cells for targeted antimicrobial treatment

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Abstract

Infectious diseases continue to pose a serious threat to human health as a result of the spread of antibiotic resistance, underscoring the urgent need for new and focused therapeutic approaches. Due to their regenerative and immunomodulatory capabilities, stem cells have emerged as a potential source for the development of antimicrobial therapies. This paper reviews the potential of stem cells as a targeted strategy for combating infections, focusing on their ability to differentiate into specific cell types that can directly target and eliminate microorganisms, as well as their capacity to modulate the immune response and enhance host defenses. The article discusses the challenges and opportunities associated with the clinical implementation of antimicrobial therapies derived from stem cells. Among these obstacles are the need for uniform protocols for cell isolation, expansion, and delivery, as well as the significance of rigorous evaluations of safety and efficacy. Despite that, the application of stem cells as a targeted antimicrobial approach holds significant potential for the development of effective and enduring therapeutic interventions for infectious diseases.

Keywords: Stem cells; Antimicrobial therapy; Targeted delivery; Regenerative medicine; Host defense mechanisms

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1. Introduction

Infectious illnesses continue to pose a serious danger to global health, significantly increasing the morbidity and mortality rates everywhere. Despite substantial advancements in sanitation, antimicrobial medication research, vaccination, and infection management over the past century, emerging and re-emerging pathogens continue to be a major concern. It has become more challenging to treat infections using currently available medications as a result of the escalation of antibiotic resistance. The World Health Organization estimates that at least 700,000 people die each year from drug-resistant illnesses, and this fatality rate will reach 10 million by 2050 if no effective measures are taken to tackle this issue[1]. To address the growing issue of antimicrobial resistance and improve outcomes for patients with infectious diseases, there is an urgent need for novel and focused treatment alternatives. Due to their special qualities that make them suitable for focused antimicrobial treatment, stem cell-based therapies have emerged as a viable approach to tackling this problem. Utilizing stem cells capacity for regeneration

and immunomodulation, it may be able to create brand-new treatments that efficiently target and destroy microbes while causing the least amount of harm to host tissues[2].

In low- and middle-income nations with limited access to healthcare and resources for disease control and prevention, infectious illnesses continue to be a considerable burden in addition to the threat posed by antibiotic resistance. For instance, millions of people continue to be affected by illnesses such as tuberculosis, malaria, and HIV/AIDS throughout the world, especially in areas with scarce resources[3]. To address these global health issues, there is a need for creative and economical treatment approaches that can be scaled up. By providing a tailored and individualized approach to antimicrobial treatment, stem cell-based therapies have the potential to solve some of these issues. The ability to develop and differentiate stem cells into particular cell types that can specifically target and eradicate microbial pathogens allows for the isolation of stem cells from either the patient's own tissues or from a donor. They are especially desirable for treating infectious disorders linked to immunological dysfunction because they can modify the immune response and strengthen host defensive mechanisms[4]. For addressing the problems with infectious diseases and antimicrobial resistance that threaten global health, stem cell-based therapies for antimicrobial treatment hold significant potential. To improve these treatments and guarantee their safety and efficacy, substantial research and development is still required.

Due to their unique characteristics that make them optimal for this application, stem cells have emerged as a potential source for the development of new and targeted treatments for infectious diseases. One of the primary characteristics of stem cells is their ability to differentiate into specialized cell types that can specifically target and eradicate microorganisms. It has been shown that mesenchymal stem cells (MSCs) can differentiate into macrophages, which are vital immune cells that can engulf and eradicate invasive infections[5]. Stem cells can differentiate, but they also possess immunomodulatory properties that can enhance the immune response of the host and promote the healing of infections. MSCs have been shown to regulate the production of cytokines and chemokines, which are crucial signaling molecules that regulate the immune response. MSCs can also inhibit the activity of immune cells that cause tissue injury and inflammation to reduce the severity of infections.

Antimicrobial peptides (AMPs), which are petite proteins of natural occurrence, play a crucial role in safeguarding the human body from various pathogenic agents. Scientists have extended this concept by synthesizing AMPs that mimic the actions of their natural counterparts[6]. The synthetic AMPs have showcased remarkable efficacy in neutralizing harmful microorganisms. MSCs, which are present in various tissues such as bone marrow and adipose tissue, hold the potential for producing a diverse range of bioactive molecules, including AMPs. The fundamental idea of this treatment strategy is to genetically modify MSCs to synthesize and secrete synthetic AMPs. On administering to a patient, these engineered MSCs can continually release the synthetic AMPs, fortifying the innate defense mechanism of the body against infections.

Moreover, stem cells, particularly MSCs, can serve as delivery vehicles for antimicrobial drugs. Researchers can achieve targeted drug delivery by loading these cells with antimicrobial agents and guiding their migration toward infected areas through chemical signals. This localization enhances the drug's concentration precisely at the infection site, optimizing its efficacy while minimizing systemic side effects. However, this pioneering concept comes with certain challenges and limitations. Ensuring consistent production of synthetic AMPs by MSCs demands intricate genetic engineering techniques, and the long-term safety implications of introducing genetically modified cells into patients need to be rigorously evaluated. In addition, directing stem cells to exclusively target infected tissues, without affecting healthy ones, remains a complex endeavor[7].

In addition, stem cells may function as a focused delivery route for antimicrobial substances[8]. It may be possible to develop a highly targeted and localized therapy that may directly eradicate microbial agents without hurting host tissues by engineering stem cells to express AMPs or antibodies. Due to the tremendous potential of stem cells as a targeted strategy against infections, there is a growing interest in developing stem cell-based therapeutics for a variety of infectious disorders[9]. Significant research and development are still required to enhance these treatments and ensure their safety and effectiveness. In the sections that follow, we will discuss in greater depth the mechanisms underlying the antimicrobial activity of stem cells, as well as the challenges and opportunities associated with the clinical application of stem cell-based antimicrobial therapy.

Compared to conventional medications, which frequently target general elements of microbial physiology and can have considerable off-target effects on host cells, stem cells offer a novel strategy for antimicrobial therapy. On the other hand, stem cells can be modified to target certain pathogens or infected host cells, making them a highly targeted and customized therapy. In addition, stem cells have the capacity to encourage tissue regeneration

and repair, which is crucial in situations where infections have severely harmed the host tissues[10]. Delivering stem cells to the infection site may make it possible to stimulate tissue healing and restoration in addition to eradicating the microbial invaders.

Antimicrobial resistance is a problem that stem cell therapy may be able to address. Since stem cells can specialize into specific cell types, they may be able to be engineered to produce antimicrobial medications that circumvent resistance mechanisms. It has been demonstrated that a synthetic AMP produced by MSCs can effectively eradicate drug-resistant bacteria *in vitro* and in animal models[10]. Due to the tremendous potential of stem cells as a targeted strategy against infections, there is a growing interest in developing stem cell-based therapeutics for a variety of infectious disorders. Due to their unique characteristics, such as differentiation potential, immunomodulatory abilities, and targeted delivery capacities, stem cells present a promising avenue for the development of novel and efficacious antimicrobial therapies.

2. Stem cell properties relevant to antimicrobial therapy

Due to their capacity for differentiation, regenerative and immunomodulatory abilities, and capacity to deliver antimicrobials to particular areas, stem cells are highly advantageous for antimicrobial therapy. Stem cells may self-renew and develop into a number of different types of specialized cells. Stem cells are particularly appealing for creating treatments that may replace or repair damaged tissues after an infection due to their capacity to regenerate[11]. For example, stem cells have been shown to promote tissue regeneration in animal models of pneumonia and lung injuries. In addition to their regenerative capabilities, stem cells also possess immunomodulatory properties that can help regulate the immune system and accelerate the healing of infections. Cytokines and chemokines are essential signaling molecules that regulate the immune response, and stem cells have the capacity to alter their production[12].

Stem cells can specialize into particular cell types that can specifically target and eradicate microorganisms. It has been shown that MSCs can differentiate into macrophages, which are vital immune cells that can engulf and eliminate invasive infections[13]. It has been shown that neural stem cells can differentiate into neurons capable of combating viral infections in the brain. Stem cells can be used to transport antimicrobial medications to specific body regions. It may be possible to develop a highly targeted and localized therapy that may directly eradicate microbial agents without hurting host tissues by engineering stem cells to express AMPs or antibodies[14]. In addition, stem cells can be modified to specifically target infected host cells such as immune or endothelial cells. Numerous characteristics of stem cells make them particularly pertinent for antimicrobial therapy. These include their capacity for regeneration and immunomodulation, as well as their capacity for differentiation and capacity to deliver antimicrobial drugs to particular sites. These distinct qualities make stem cells a promising source for the creation of fresh and efficient antimicrobial remedies[15].

The capacity to self-renew and differentiate into a range of specialized cell types is a property of stem cells. They are especially appealing for developing medicines that can repair or replace damaged tissues after infection due to their regeneration capacity. Depending on their source and lineage, stem cells can develop into a variety of cell types[16]. For instance, MSCs can differentiate into bone, cartilage, and fat cells, whereas hematopoietic stem cells produce red and white blood cells. Neurons and glial cells, which are significant cell types in the central nervous system, can develop from neural stem cells.

It has been demonstrated that stem cells support tissue regeneration and restoration in the context of antimicrobial therapy. In animal models of pneumonia and lung injury, stem cells have been shown to enhance lung function and promote tissue repair. Stem cells have been shown to lessen cerebral inflammation and promote neuronal regeneration in animal models of bacterial meningitis. In addition, stem cells have the ability to secrete cytokines and growth factors that aid in tissue regeneration and repair[17]. For instance, it has been shown that MSCs release molecules that encourage the growth of blood vessels and tissue healing. Depending on the type and stage of the infection as well as the unique characteristics of the stem cells being used, the impact of stem cells on tissue repair and regeneration may differ[18]. Additional study is necessary to completely understand stem cells' ability to regenerate in the context of antimicrobial therapy.

The immunomodulatory abilities of stem cells can help control the immune system and eliminate infections. Due to these qualities, stem cells are particularly appealing for the creation of anti-infection treatments that lessen tissue damage and inflammation. The production of cytokines and chemokines, which are crucial signaling molecules that control the immune response, can be changed by stem cells[19,20]. By secreting substances that promote an anti-inflammatory response, stem cells can reduce the severity of infections and prevent tissue injury. In animal models of sepsis, MSC therapy has been shown to reduce inflammation and increase survival rates[21]. In addition, stem cells can inhibit immune cells that cause tissue injury

and inflammation. It has been demonstrated, for instance, that MSCs inhibit the function of T-cells, a key immune cell that can cause tissue injury in autoimmune and inflammatory diseases. By reducing the activity of these cells, MSCs can aid in the prevention of tissue injury and the promotion of tissue healing.

Stem cells have anti-inflammatory properties, but they can also boost the host immune system and aid in infection eradication. The cytokines and chemokines that draw immune cells to the site of infection can be induced by stem cells. For instance, it has been demonstrated that therapy with neural stem cells promotes recruitment of immune cells to the brain in animal models of viral infections, thereby improving viral clearance. The immunomodulatory effects of stem cells can, however, differ depending on the type and stage of infection as well as the unique properties of the stem cells being employed[22]. To completely comprehend the immunomodulatory potential of stem cells in the context of antimicrobial therapy, more study is warranted.

Depending on their origin and ancestry, stem cells can differentiate into a variety of specialized cell types. Due to their capacity for differentiation, stem cells are particularly attractive for the development of medications that can specifically target and eliminate microbes. It has been shown that MSCs can differentiate into macrophages, which are vital immune cells that can engulf and eliminate invasive infections[23]. MSCs are capable of differentiating into osteoblasts and chondrocytes, the two cell types that can directly target microbes and assist in bone and cartilage regeneration after infections.

It has been demonstrated that neural stem cells can develop into neurons that can fight viral infections in the brain. Neural stem cell transplantation has been demonstrated in animal models of viral encephalitis to induce the development of new neurons that can specifically target the virus and eradicate the infection. Stem cells can be designed to distribute antimicrobial drugs to certain sites in addition to their direct antimicrobial actions[24]. It may be conceivable to develop a highly targeted and localized therapy that may directly eradicate microbial agents without affecting host tissues by engineering stem cells to express AMPs or antibodies. The differentiation capacity of stem cells might, however, change based on their source and lineage as well as the particular properties of the stem cells being used. To completely comprehend the potential of stem cell differentiation in the context of antimicrobial therapy, more study is required.

Stem cells may be used to deliver antimicrobial drugs to specific areas of the body[25]. It may be possible to develop a highly targeted and localized therapy that may directly eradicate microbial agents without hurting host tissues by engineering stem cells to express AMPs or antibodies. For instance, scientists have altered MSCs to express a synthetic AMP that has been shown to successfully eradicate germs both *in vitro* and in animal infection models. The AMP was effectively delivered to the bacterial cells by the modified MSCs, which enabled them to move to the infection site.

Stem cells can be designed to specifically target infected host cells in addition to delivering antimicrobial agents to microbial cells directly. For instance, it has been demonstrated that MSCs move to the brain and particularly target inflamed blood arteries in animal models of bacterial meningitis. The MSCs were able to lessen inflammation and encourage bacterial clearance by administering therapeutic medicines directly to the affected arteries[26]. In addition, stem cells can be genetically modified to target particular cell-surface markers that are increased in response to infection. For instance, a protein that precisely targets and attaches to inflamed arteries has been designed and incorporated into MSCs. In animal models of bacterial meningitis, the MSCs were able to increase bacterial clearance and lessen inflammation by accumulating in these arteries[27]. The release of antimicrobial drugs using stem cells may be highly targeted and individualized. It may be possible to build cutting-edge and potent antimicrobial medicines that limit harm to host tissues by engineering stem cells to express particular therapeutic molecules and target specific cells or tissues damaged by infection.

Stem cells possess distinct characteristics that render them potentially valuable for conveying medicinal drugs to particular regions of the body, including infected sites[28]. Through chemical signals, stem cells can be steered toward specific tissues or areas, and this quality can be exploited to direct them to infected regions. The concept of utilizing stem cells, particularly MSCs, as conveyance mechanisms for antimicrobial drugs is being investigated by scientists. This process entails loading the stem cells with antimicrobial drugs and then administering them to the patient. Once present in the body, the stem cells can be directed to migrate toward the infected site due to the presence of signals discharged by the infected tissue. The stem cells can, then, discharge the antimicrobial drugs directly at the infected site, which can enhance the drug's local concentration and efficacy.

3. Mechanisms of stem cell antimicrobial activity

Stem cells are capable of exhibiting direct antimicrobial properties as a result of their capacity to differentiate into distinct cell lineages that can specifically identify and eliminate microbial pathogens. MSCs are capable

of differentiating into macrophages, which are essential immune cells responsible for phagocytosis and elimination of foreign pathogens[29]. In addition, stem cells have the ability to produce and release AMPs and proteins, which can directly inhibit the proliferation of microorganisms or cause their demise. Peptides and proteins are capable of disrupting the cell membranes of microorganisms, impeding the replication of microbial DNA, preventing protein synthesis, and interfering with the physiology of micro-organisms as a whole.

The ability of human embryonic stem cells to produce AMPs with inhibitory effects against bacteria, fungi, and viruses has been demonstrated[30]. However, the ability of defensins, cathelicidins, and histatins to inhibit microbial growth and disrupt microbial cell membranes remain contentious. Similarly, MSCs are capable of releasing cathelicidin, an AMP that eliminates microbes by interfering with their cell membranes[31]. It is possible to manipulate stem cells to generate artificial AMPs that target specific microorganisms. For example, scientists have manipulated MSCs to generate a synthetic AMP that is effective against methicillin-resistant *Staphylococcus aureus*, a variant of *S. aureus* resistant to many conventional antibiotics. The modified MSCs showed efficient bactericidal action against methicillin-resistant *S. aureus* in both *in vitro* and *in vivo* animal infection models[32]. Nonetheless, the antibacterial abilities of stem cells are complex and situational.

As compact protein molecules, AMPs perform a vital function in the immune defense mechanism of the body by combating various pathogens, such as bacteria, viruses, and fungi. Immune cells represent the natural source of these peptides. Driven by the desire for more controllable production of AMPs, recently, there has been a surge in the studies regarding the feasibility of artificially synthesizing AMPs as potential therapeutic agents. These peptides are designed to imitate natural AMPs and can exterminate or hinder the proliferation of harmful microorganisms. The human body houses a store of MSCs, which are present in different tissues such as adipose and bone marrow. MSCs can generate a range of bioactive molecules, including AMPs. Researchers are studying the concept of genetically engineering MSCs to generate artificial AMPs[33]. The introduction of these engineered MSCs to patients could pave the way for a novel therapeutic approach in combating infections. These cells would continuously synthesize and discharge synthetic AMPs, amplifying the body's capability to fight infections.

Stem cells have the ability to alter the host's immune response in a number of ways, which helps in the treatment of infections. Stem cells have the capacity to control cytokine and chemokine production, promoting an anti-inflammatory response, and reducing tissue damage brought on by inflammation. It has been noted that immune cells that cause tissue inflammation and injury can be inhibited by stem cells. T-cells, a crucial component of immune cells that significantly contribute to tissue injury in autoimmune and inflammatory disorders, can be inhibited in their activity by MSCs[34]. MSCs have the capacity to reduce tissue damage and enhance tissue regeneration by regulating the activity of these cells. Activation of MSC using Toll-like receptor (TLR) ligands exhibited a noteworthy augmentation in the generation of the AMP called CXCL10, while simultaneously suppressing bacterial proliferation in an artificial environment. Among the TLR agonists examined *in vitro*, poly(I:C), a TLR3 agonist, displayed the greatest efficacy. In the context of chronic *S. aureus* infection, MSC activation with poly(I: C) elicited a reduction in the quantitative assessment of bacterial presence[35].

In addition, stem cells can promote the development of regulatory T-cells, which are essential for maintaining immunological homeostasis and preventing unnecessary immune activation. Regulatory T-cells have the ability to inhibit the function of other immune cells that participate in the inflammatory response and cause harm to tissues, while also facilitating the process of tissue repair. MSCs have demonstrated the ability to enhance the generation of regulatory T-cells and mitigate inflammation in animal models of bacterial pneumonia, resulting in enhanced bacterial elimination and pulmonary performance[36]. Studies conducted on animal models of viral encephalitis have demonstrated that the transplantation of neural stem cells can effectively mitigate inflammation and facilitate the regeneration of neurons. The manipulation of the host's immune response through stem cells presents a hopeful direction for the advancement of innovative and efficacious antimicrobial treatments[37]. Stem cells have the potential to regulate infections and facilitate tissue regeneration by inducing an anti-inflammatory response and mitigating tissue damage. Systemic administration of activated MSCs to mice with pre-existing *S. aureus* biofilm infections resulted in a notable reduction in bacterial quantities at the site of the wound, thus leading to an enhancement in wound healing, particularly when executed in conjunction with antibiotic treatment[38].

Table 1 provides an organized breakdown of the key steps involved in the mechanism of synthetic AMP production within MSCs. It highlights how genetic modification, transcription, translation, and cellular processes contribute to the production and release of AMPs, ultimately leading to the antimicrobial effect. The activation of cytokines and chemokines by stem cells can augment the immune response of the host, leading to the recruitment of immune cells to the location of infection.

Table 1. Mechanism of synthetic antimicrobial peptide production within mesenchymal stem cells

Mechanism of synthetic AMP production within MSCs	Description and explanation	Key factors and considerations	Reference
Genetic modification	MSCs are genetically engineered to express synthetic AMP genes, typically using viral vectors or CRISPR-Cas9 technology.	Selection of appropriate gene delivery methods	Cheng *et al*., 2021[39]
Transcription and translation	The synthetic AMP gene is transcribed into mRNA in the cell nucleus. The mRNA is, then, transported to the cytoplasm, where ribosomes translate it into AMPs.	Regulation of gene expression levels	Weis *et al*., 2013[40]
Post-translational modifications	Newly synthesized AMPs undergo post-translational modifications, such as signal peptide cleavage, folding, and potential glycosylation.	Ensuring proper folding and functional modifications	Tiwari, 2019[41]
Storage and secretion	Processed AMPs are stored in secretory vesicles, such as exosomes, and are transported to the cell membrane for release.	Optimization of vesicle secretion pathways	Verkhratsky *et al*., 2016[42]
Local immune activation	Released synthetic AMPs interact with pathogens by disrupting their membranes or interfering with intracellular processes.	Selection of AMPs with broad-spectrum activity	Wang *et al*., 2019[43]
Continuous production	Engineered MSCs sustain synthetic AMP production, resulting in a continuous release of AMPs for extended antimicrobial effects.	Monitoring long-term AMP production stability	Rai *et al*., 2022[44]
Site-specific migration	Engineered MSCs can be guided to migrate toward infection sites through chemotactic signals, enhancing localized AMP delivery.	Designing strategies for effective MSC migration	Zi *et al*., 2022[45]
Regulatory considerations	Genetically modified MSCs must meet safety and regulatory standards for clinical applications, ensuring patient well-being.	Compliance with ethical and regulatory guidelines	Zocchi, 2019[46]

Abbreviations: AMP: Antimicrobial peptide; MSC: Mesenchymal stem cells.

Stem cells have the potential to enhance the recruitment of immune cells, thereby aiding in the eradication of microbial agents and the regulation of infections[47]. In animal models pertaining to viral infections, the administration of neural stem cells has demonstrated the ability to induce the mobilization of immune cells toward the brain, thereby resulting in enhanced viral elimination. In animal models of bacterial meningitis, MSCs have been shown to improve the recruitment of immune cells to the infection site, leading to increased bacterial clearance[48]. In addition to their function in promoting immune cell recruitment, stem cells can also enhance the activity of immune cells. Analysis of the expression of major AMPs revealed that one of the contributing factors to the antimicrobial activity of MSC-conditioned medium against Gram-negative bacteria was the presence of the human cathelicidin AMP, hCAP-18/LL-37. Both the mRNA and protein expression data illustrated an increase in LL-37 expression in MSCs following bacterial challenge. By employing an *in vivo* mouse model of *E. coli pneumonia*, the intratracheal administration of MSCs resulted in a reduction in bacterial growth, as measured by colony-forming units, in both the lung homogenates and the bronchoalveolar lavage fluid. Furthermore, when MSCs were administered concurrently with a neutralizing antibody to LL-37, there was a noticeable decrease in bacterial clearance[49].

MSC injection has been shown to improve neutrophil performance in animal models of bacterial infections. Neutrophils are an essential subset of immune cells that phagocytose and destroy invading pathogens. Human embryonic stem cells have been shown to stimulate the production of interferon alpha in animal models of viral infections, which inhibits viral replication and improves viral clearance[50]. A promising method for the creation of novel and effective antimicrobial therapies is stem cell-mediated host immunity enhancement. Stem cells have the capacity to control immune cell generation and activity, making it easier to treat infections and improve microbial clearance.

4. Challenges and opportunities for clinical translation

The development of stem cell-derived treatments for antimicrobial therapy is hampered by the lack of standard techniques for stem cell extraction, proliferation, and delivery[51]. The therapeutic potential of stem cell-based therapies may be constrained by variance in the aforementioned processes, which may affect stem cell quality and efficacy. Because different isolation methods are used, there might be variation in the quality and effectiveness of the stem cells obtained from different tissues and sources. The extraction of MSCs from either bone marrow or adipose tissue may lead to varying degrees of stem cell purity and differentiation capacity.

The proliferation of stem cells *in vitro* is a prerequisite for achieving a sufficient number of cells for therapeutic purposes. The quality and efficacy of stem cells can be

influenced by the conditions and protocols employed for their expansion. Variations in culture media, growth factors, and cell density may lead to variations in the proliferation and differentiation potential of stem cells. The transportation of stem cells to the location of infection is a crucial factor to be taken into account in stem cell-oriented treatments for combating microbial infections. The selection of the mode of administration may impact the feasibility and efficacy of stem cells. An instance of stem cell accumulation in the lungs can be observed following intravenous injection, whereas stem cell retention at the injection site can be observed following direct injection[52]. The implementation of standardized protocols for the isolation, expansion, and delivery of stem cells can promote uniformity in stem cell quality and effectiveness across diverse laboratory and clinical environments. The facilitation of the development and testing of stem cell-based therapies for antimicrobial treatment can enhance the translational potential of stem cell research into clinical practice. Furthermore, the implementation of uniform procedures can contribute to safeguarding the well-being of patients undergoing stem cell treatments, through the reduction of potential harmful incidents and the assurance of consistent clinical results.

Table 2 provides a clear overview of the challenges associated with achieving consistent AMP production by MSCs, highlighting the complexities and factors that researchers need to address for the successful implementation of this therapeutic approach. Preclinical investigations have demonstrated the potential of antimicrobial therapies based on stem cells[60]. However, the implementation of these therapies in clinical settings necessitates meticulous examination to ascertain their safety and effectiveness. The implementation of safety testing is imperative for reducing the likelihood of unfavorable occurrences linked to stem cell therapy. Similarly, efficacy testing is essential to assess the potency of stem cell-derived antimicrobial interventions. The assessment of safety for antimicrobial therapies that utilize stem cells entails the examination of potential hazards arising from the transplantation of stem cells[61]. The aforementioned hazards encompass the possibility of immunological rejection, neoplastic growth, and dissemination of pathogenic microorganisms.

Autologous transplantation, which utilizes the patient's own tissues, or allogeneic transplantation, which involves obtaining stem cells from a donor with a matching genetic profile, may be utilized to reduce the risk of immune rejection. In addition, it is possible to manipulate stem cells to express immunosuppressive agents, thereby reducing the likelihood of immune rejection. To reduce the probability of tumorigenesis, it is essential to screen stem cells for mutations or chromosomal aberrations that may increase the likelihood of tumorigenesis[62]. Stem cells can also be manipulated to express tumor suppressor genes or other components that lessen the likelihood of tumor development. Screening stem cells for potential infectious agents and cultivating and storing them in a sterile environment are necessary to lower the risk of infectious agent transmission. Analyzing stem cell-based antimicrobial therapies' capacity to control infections and encourage tissue regeneration is necessary to evaluate their effectiveness[63]. To complete this endeavor, preclinical animal models and clinical trials on humans can be utilized.

The efficacy of stem cell-based antimicrobial therapies in controlling infections, reducing inflammation, and

Table 2. Discussion of challenges in consistent antimicrobial peptides production by mesenchymal stem cells

Challenges in consistent AMP production by MSCs	Description and explanation	References
Complexities in genetic engineering	Genetic modification of MSCs to produce synthetic AMPs requires sophisticated techniques to ensure reliable and efficient production	Herrmann *et al.*, 2021[53]
Variability in MSC responses	MSCs derived from different sources or individuals might exhibit varying production capabilities, affecting treatment consistency	Dunn *et al.*, 2021[54]
Regulation of AMP expression	Achieving precise control over the levels and timing of synthetic AMP expression by MSCs is challenging, influencing treatment efficacy.	Zhang *et al.*, 2022[55]
Long-term stability of AMP production	Ensuring sustained AMP production over extended periods without diminishing efficacy remains a technical hurdle	Teixeira *et al.*, 2020[56]
Immune response to engineered MSCs	The introduction of genetically modified cells could trigger an immune response, potentially impacting treatment safety and effectiveness	Gao *et al.*, 2022[57]
Optimization of synthetic AMP design	Designing synthetic AMPs that are both effective against a range of pathogens and compatible with MSC production processes is an intricate process	Chen *et al.*, 2022[58]
Microenvironment influence	The local microenvironment at the infection site can impact MSC behavior and AMP production, affecting treatment consistency	Zhang *et al.*, 2022[59]

Abbreviations: AMP: Antimicrobial peptide; MSC: Mesenchymal stem cells.

promoting tissue healing can be assessed in animal models. The aforementioned studies have the potential to furnish significant insights regarding the safety and effectiveness of stem cell treatments before their clinical trials on human subjects[64]. The efficacy of stem cell-derived antimicrobial therapies in the treatment of human infections can be assessed through clinical trials. Clinical trials can offer significant insights into the safety and effectiveness of stem cell treatments in human subjects, thereby contributing to the advancement of stem cell-based antimicrobial therapies[65]. The process of developing and translating stem cell-based antimicrobial therapies necessitates the crucial undertaking of safety and efficacy testing. By guaranteeing the safety and effectiveness of these therapies, it is possible to create new and efficient treatments for infections that exhibit resistance to conventional antibiotics.

Stem cell-based antimicrobial therapies are subject to regulatory oversight by government agencies such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency[66]. The aforementioned organizations are responsible for monitoring the development, production, and medical application of stem cell-based therapies to ensure their safety and efficacy. Stem cell-based antimicrobial medicines are governed by the FDA in the U.S. as biological products. To guarantee the consistency and excellence of the manufacturing processes, the FDA mandates that the aforementioned items be produced in compliance with the current good manufacturing practices[67]. The FDA also mandates that clinical trials for stem cell-based treatments be carried out under an investigational new drug application. Data proving the safety and effectiveness of the therapy must be submitted to do this.

The issue of scalability in manufacturing holds significant importance in the context of the advancement of antimicrobial therapies based on stem cells. As these therapeutic interventions transition from preclinical stages to clinical trials and eventual commercialization, it becomes imperative to scale up their production to cater to the growing demand for treatment[68]. The concept of manufacturing scalability pertains to the creation of procedures and innovations that can be expanded to generate substantial volumes of stem cells, while ensuring the preservation of their quality and effectiveness. The optimization of protocols for stem cell isolation, expansion, and delivery is necessary to ensure their efficient and consistent performance on a large scale.

The manufacturing scalability of stem cell-based antimicrobial therapies is hindered by the potential variability in stem cell quality and efficacy that may arise during the process of large-scale production. Disparities in cultural milieu, growth stimulants, and cellular concentration may give rise to variations in the caliber and potency of stem cells, thereby constraining the efficaciousness of stem cell treatments. In response to this challenge, scholars are currently devising technological solutions to mechanize and regulate the production procedures of stem cells[69]. The utilization of bioreactors, microcarrier systems, and closed-system processing has been shown to enhance the uniformity and efficacy of stem cell manufacturing.

Furthermore, investigators are examining the potential of allogeneic stem cells, which can be generated in significant quantities from a solitary donor and employed to address numerous patients. The utilization of allogeneic stem cells has the potential to decrease the requirement for autologous stem cells, which necessitate extraction from the patient's own tissues and can be a laborious and expensive process[70]. The scalability of manufacturing is a crucial factor to be taken into account in the advancement and implementation of antimicrobial therapies based on stem cells. Through the optimization of stem cell manufacturing procedures and the advancement of technologies that automate and standardize these procedures, it is possible to generate significant quantities of stem cells while preserving their quality and effectiveness.

5. Current and future applications of stem cell antimicrobial therapy

Insights into the safety and efficacy of stem cell-based antimicrobial therapies have been obtained through preclinical and clinical studies. Various investigations have examined diverse stem cell varieties, modes of administration, and antimicrobial objectives to establish innovative and efficacious therapies for antibiotic-resistant infections. An instance of a preclinical investigation involves the utilization of MSCs for the treatment of bacterial infections. MSCs have demonstrated the ability to mitigate inflammation, enhance bacterial elimination, and facilitate tissue regeneration in animal models of bacterial infections[71,72]. It is believed that the impacts are facilitated through the immunomodulatory and antimicrobial characteristics of MSCs.

An instance of preclinical research involves the utilization of neural stem cells for the purpose of treating viral infections. Studies conducted on animal models have demonstrated that neural stem cells possess the ability to facilitate viral clearance and mitigate inflammation in the brain during viral infections. It is believed that the immunomodulatory and neuroprotective properties of neural stem cells are responsible for these observed effects. Apart from preclinical investigations, human clinical trials have been carried out to assess the safety and effectiveness

of antimicrobial therapies based on stem cells[73]. A clinical trial of phase I was executed to assess the safety and effectiveness of intravenous infusion of allogeneic MSCs in patients diagnosed with severe sepsis. The research discovered that the infusion of MSCs was deemed safe and well-tolerated and was linked to enhanced survival rates and decreased organ dysfunction. Another example of a clinical study is the use of neural progenitor cells produced from human embryonic stem cells to treat herpes simplex virus (HSV)-induced encephalitis[74]. Individuals with HSV encephalitis were given intracerebral injections of neural progenitor cells generated from human embryonic stem cells during a phase I clinical trial. The study showed that the treatment was safe, well-tolerated, and associated with improved neurological outcomes. Case studies and examples of preclinical and clinical investigations illustrate the promise of stem cell-based antimicrobial therapy as unique and successful treatments for infections that are resistant to conventional antibiotics. Through the examination of diverse stem cell varieties, modes of delivery, and antimicrobial objectives, it is possible to devise customized remedies for different infection types and promote the development and application of stem cell-based antimicrobial interventions.

The utilization of stem cell-based antimicrobial therapies is a burgeoning field that shows great potential for the creation of innovative and efficacious remedies for infections resistant to conventional antibiotics. The latest developments in stem cell research have created new prospects for the creation of specific antimicrobial treatments through the utilization of stem cells[75]. An emerging prospect involves the utilization of genetically modified stem cells to manifest AMPs or other elements that can amplify the antimicrobial characteristics of stem cells. Through the expression of these factors, stem cells may enhance their efficacy in managing infections and facilitating tissue regeneration. An additional developing prospect pertains to the utilization of stem cells for administering precise antimicrobial agents to the specific location of infection. It is possible to manipulate stem cells to secrete antimicrobial agents on detection of particular stimuli, such as the existence of bacterial or viral antigens. The precise administration of antimicrobial agents can enhance their efficacy and mitigate the potential for non-specific impacts[76].

Various types of stem cells possess differing capabilities in generating and distributing antimicrobial agents. One example is MSCs, which have been extensively researched for their immunomodulatory properties and potential to generate AMPs. However, the effectiveness of the antimicrobial effect may differ depending on the specific type of AMP generated, the concentration of the peptide, and the microenvironment of the site of infection. The production of synthetic AMPs by MSCs may not be uniform across all cells and may necessitate meticulous genetic engineering techniques. The introduction of genetically modified cells into a patient's body may trigger an immune response or result in unwanted side effects. Although stem cells can be guided to migrate toward specific regions, ensuring that they solely target infected tissues and not healthy ones poses a challenge. The long-term safety of using engineered stem cells and synthetic peptides in humans is still under investigation[77]. The development and implementation of this approach necessitate advanced biotechnological processes and rigorous safety evaluations. The genetic alteration of stem cells raises ethical concerns that must be thoughtfully addressed.

Furthermore, researchers are investigating the potential of stem cells in the creation of immunizations for contagious illnesses. Stem cells have the ability to produce large amounts of viral or bacterial antigens, evoking the host immune response, and promoting the development of protective immunity against pathogenic micro-organisms[78]. In addition, ongoing research investigates the ability of stem cells to regulate the microbiome, which is the collection of microorganisms that inhabit the human body. The occurrence of dysbiosis, which refers to an imbalance in the microbiome's composition, has been linked to numerous infectious and inflammatory diseases. Stem cells have the potential to modulate the microbiome by promoting the proliferation of beneficial micro-organisms and inhibiting the proliferation of harmful microorganisms. Targeted antimicrobial therapies utilizing stem cells represent a potential avenue for the development of innovative and effective therapeutics for antibiotic-resistant infections. The utilization of stem cells' distinctive characteristics can facilitate the creation of customized remedies for diverse categories of infections and promote the development of stem cell-oriented antimicrobial therapies.

6. Conclusion

The area of research pertaining to the potential of stem cells for targeted antimicrobial treatment is a rapidly evolving and exciting field. Due to their ability to differentiate into specialized cellular lineages and control the immune system's response, stem cells have unique properties that make them particularly well-suited for treating infectious diseases. The development of stem cell-based antimicrobial treatments has the potential to revolutionize the way we treat infections, despite the existence of a number of issues that need to be addressed, such as the creation of standardized procedures for cell isolation, expansion, and delivery. In response to the escalating antibiotic resistance crisis, development of novel and targeted therapeutic

alternatives become an imperative endeavor. Given our understanding about the versatility of stem cells, they offer a potential solution for this critical public health issue. Thus, future research on stem cells should focus on unleashing their application potential for targeted antimicrobial therapy.

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References

1. De Kraker ME, Stewardson AJ, Harbarth S, 2016, Will 10 million people die a year due to antimicrobial resistance by 2050? *PLoS Med*, 13(11): e1002184.

   https://doi.org/10.1371/journal.pmed.1002184

2. Wu HH, Zhou Y, Tabata Y, *et al*., 2019, Mesenchymal stem cell-based drug delivery strategy: From cells to biomimetic. *J Control Release*, 294: 102–113.

   https://doi.org/10.1016/j.jconrel.2018.12.019

3. Vitoria M, Granich R, Gilks CF, *et al*., 2009, The global fight against HIV/AIDS, tuberculosis, and malariacurrent status and future perspectives. *Am J Clin Pathol*, 131(6): 844–848.

   https://doi.org/10.1309/AJCP5XHDB1PNAEYT

4. Medzhitov R, Schneider DS, Soares MP, 2012, Disease tolerance as a defense strategy. *Science*, 335(6071): 936–941.

   https://doi.org/10.1126/science.1214935

5. Seo N, Akiyoshi K, Shiku H, 2018, Exosome-mediated regulation of tumor immunology. *Cancer Sci*, 109(10): 2998–3004.

   https://doi.org/10.1111/cas.13735

6. Giuliani A, Rinaldi AC, 2011, Beyond natural antimicrobial peptides: Multimeric peptides and other peptidomimetic approaches. *Cell Mol Life Sci*, 68: 2255–2266.

7. Xu XY, Li X, Wang J, *et al*., 2019, Concise review: Periodontal tissue regeneration using stem cells: Strategies and translational considerations. *Stem Cells Transl Med*, 8(4): 392–403.

   https://doi.org/10.1002/sctm.18-0181

8. Sun H, Zhang T, Gao J, 2022, Extracellular vesicles derived from mesenchymal stem cells: A potential biodrug for acute respiratory distress syndrome treatment. *BioDrugs*, 36(6): 701–715.

   https://doi.org/10.1007/s40259-022-00555-5

9. Pellegrino E, Gutierrez MG, 2021, Human stem cell-based models for studying host-pathogen interactions. *Cell Microbiol*, 23(7): e13335.

   https://doi.org/10.1111/cmi.13335

10. Shang F, Yu Y, Liu S, *et al*., 2021, Advancing application of mesenchymal stem cell-based bone tissue regeneration. *Bioact Mater*, 6(3): 666–683.

    https://doi.org/10.1016/j.bioactmat.2020.08.014

11. Volponi AA, Pang Y, Sharpe PT., 2010, Stem cell-based biological tooth repair and regeneration. *Trends Cell Biol*, 20(12): 715–722.

    https://doi.org/10.1016/j.tcb.2010.09.012

12. Audette RV, Lavoie-Lamoureux A, Lavoie JP, *et al*., 2013. Inflammatory stimuli differentially modulate the transcription of paracrine signaling molecules of equine bone marrow multipotent mesenchymal stromal cells. *Osteoarthritis Cartilage*, 21(8): 1116–1124.

    https://doi.org/10.1016/j.joca.2013.05.004

13. Chen R, Hao Z, Wang Y, *et al*., 2022, Mesenchymal stem cell-immune cell interaction and related modulations for bone tissue engineering. *Stem Cells Int*, 2022: 7153584.

    https://doi.org/10.1155/2022/7153584

14. Marrazzo P, Pizzuti V, Zia S, *et al*., 2021, Microfluidic tools for enhanced characterization of therapeutic stem cells and prediction of their potential antimicrobial secretome. *Antibiotics* (*Basel*), 10(7): 750.

    https://doi.org/10.3390/antibiotics10070750

15. Deus IA, Mano JF, Custódio CA, 2020, Perinatal tissues and cells in tissue engineering and regenerative medicine. *Acta Biomater*, 110: 1–14.

    https://doi.org/10.1016/j.actbio.2020.04.035

16. Rossant J. 2008, Stem cells and early lineage development. *Cell*, 132(4): 527–531.

https://doi.org/10.1016/j.cell.2008.01.039

17. Busilacchi A, Gigante A, Mattioli-Belmonte M, *et al.*, 2013, Chitosan stabilizes platelet growth factors and modulates stem cell differentiation toward tissue regeneration. *Carbohydr Polym*, 98(1): 665–676.

https://doi.org/10.1016/j.carbpol.2013.06.044

18. Xia H, Li X, Gao W, *et al.*, 2018, Tissue repair and regeneration with endogenous stem cells. *Nat Rev Mater*, 3(7): 174–193.

https://doi.org/10.1038/s41578-018-0027-6

19. Baldridge MT, King KY, Goodell MA., 2011, Inflammatory signals regulate hematopoietic stem cells. *Trends Immunol*, 32(2): 57–65.

https://doi.org/10.1016/j.it.2010.12.003

20. Zhu Y, Yao S, Chen L., 2011, Cell surface signaling molecules in the control of immune responses: A tide model. *Immunity*, 34(4): 466–478.

https://doi.org/10.1016/j.immuni.2011.04.008

21. Lombardo E, Van der Poll T, DelaRosa O, *et al.*, 2015, Mesenchymal stem cells as a therapeutic tool to treat sepsis. *World J Stem Cells*, 7(2): 368.

https://doi.org/10.4252/wjsc.v7.i2.368

22. Le Blanc K, 2003, Immunomodulatory effects of fetal and adult mesenchymal stem cells. *Cytotherapy*, 5(6): 485–489.

https://doi.org/10.1080/14653240310003611

23. Zhang X, Kim TH, Thauland TJ, *et al.*, 2020, Unraveling the mechanobiology of immune cells. *Curr Opin Biotechnol*, 66: 236–245.

https://doi.org/10.1016/j.copbio.2020.09.004

24. Bachère E, Gueguen Y, Gonzalez M, *et al.*, 2004, Insights into the anti-microbial defense of marine invertebrates: The penaeid shrimps and the oyster *Crassostrea gigas*. *Immunological Rev*, 198(1): 149–168.

https://doi.org/10.1111/j.0105-2896.2004.00115.x

25. Bush LM., Healy CP, Javdan SB, *et al.*, 2021, Biological cells as therapeutic delivery vehicles. *Trends Pharmacol Sci*, 42(2): 106–118.

https://doi.o10.1016/j.tips.2020.11.008

26. Park J, Kim S, Lim H, *et al.*, 2019, Therapeutic effects of human mesenchymal stem cell microvesicles in an *ex vivo* perfused human lung injured with severe *E. coli* pneumonia. *Thorax*, 74(1): 43–50.

https://doi.org/10.1136/thoraxjnl-2018-211576

27. Ahn SY, Chang YS, Kim YE, *et al.*, 2018, Mesenchymal stem cells transplantation attenuates brain injury and enhances bacterial clearance in *Escherichia coli* meningitis in newborn rats. *Pediatric Res*, 84(5): 778–785.

https://doi.org/10.1136/thoraxjnl-2018-211576

28. Luarte A, Bátiz LF, Wyneken U, *et al.*, 2016, Potential therapies by stem cell-derived exosomes in CNS diseases: Focusing on the neurogenic niche. *Stem Cells İnt*, 2016: 5736059.

https://doi.org/10.1155/2016/5736059

29. Li N, Hua J, 2017, Interactions between mesenchymal stem cells and the immune system. *Cell Mol Life Sci*, 74: 2345–2360.

https://doi.org/10.1007/s00018-017-2473-5

30. Iliopoulos JM, Layrolle P, Apatzidou DA., 2022, Microbial-stem cell interactions in periodontal disease. *J Med Microbiol*, 71(4): 001503.

https://doi.org/10.1099/jmm.0.001503

31. Esfandiyari R, Halabian R, Behzadi E, *et al.*, 2019, Performance evaluation of antimicrobial peptide ll-37 and hepcidin and β-defensin-2 secreted by mesenchymal stem cells. *Heliyon*, 5(10): e02652.

https://doi.org/10.1016/j.heliyon.2019.e02652

32. Huang J, Wu S, Wu M, *et al.*, 2021, Efficacy of the therapy of 5-aminolevulinic acid photodynamic therapy combined with human umbilical cord mesenchymal stem cells on methicillin-resistant *Staphylococcus aureus*-infected wound in a diabetic mouse model. *Photodiagnosis Photodynamic Therapy*, 36: 102480.

https://doi.org/10.1016/j.pdpdt.2021.102480

33. Kurpe SR, Grishin SY, Surin AK, *et al.*, 2020, Antimicrobial and amyloidogenic activity of peptides. Can antimicrobial peptides be used against SARS-CoV-2? *Int J Mole Sci*, 21(24): 9552.

https://doi.org/10.3390/ijms21249552

34. Burr SP, Dazzi F, Garden OA, 2013, Mesenchymal stromal cells and regulatory T cells: the Yin and Yang of peripheral tolerance? *Immunol Cell Biol*, 91(1): 12–18.

https://doi.org/10.1038/icb.2012.60

35. Johnson V, Webb T, Dow S., 2013, Activated mesenchymal stem cells amplify antibiotic activity against chronic *Staphylococcus aureus* infection (P5056). *J Immunol*, 190(1 Suppl): 180.11.

https://doi.org/10.4049/jimmunol.190.Supp.180.11

36. Wang MY, Zhou TY, Zhang ZD, *et al.*, 2021, Current therapeutic strategies for respiratory diseases using mesenchymal stem cells. *MedComm* (*2020*), 2(3): 351–380.

https://doi.org/10.1002/mco2.74

37. Vivarelli S, Falzone L, Leonardi GC, *et al.*, 2021, Novel insights on gut microbiota manipulation and immune checkpoint inhibition in cancer (Review). *Int J Oncol*, 59(3): 75.

https://doi.org/10.3892/ijo.2021.5255

38. Chow L, Johnson V, Impastato R, *et al*., 2020, Antibacterial activity of human mesenchymal stem cells mediated directly by constitutively secreted factors and indirectly by activation of innate immune effector cells. *Stem Cells Transl Med*, 9(2): 235–249.

https://doi.org/10.1002/sctm.19-0092

39. Cheng H, Zhang F, Ding Y, 2021, CRISPR/Cas9 delivery system engineering for genome editing in therapeutic applications. *Pharmaceutics*, 13(10): 1649.

https://doi.org/10.3390/pharmaceutics13101649

40. Weis BL, Schleiff E, Zerges W., 2013, Protein targeting to subcellular organelles via MRNA localization. *Biochim Biophys Acta*, 1833(2): 260–273.

https://doi.org/10.1016/j.bbamcr.2012.04.004

41. Tiwari V, 2019, Post-translational modification of ESKAPE pathogens as a potential target in drug discovery. *Drug Discov Today*, 24(3): 814–822.

https://doi.org/10.1016/j.drudis.2018.12.005

42. Verkhratsky A, Matteoli M, Parpura V, *et al*., 2016, Astrocytes as secretory cells of the central nervous system: İdiosyncrasies of vesicular secretion. *EMBO J*, 35(3): 239–257.

https://doi.org/10.15252/embj.201592705

43. Wang J, Dou X, Song J, *et al*., 2019., Antimicrobial peptides: Promising alternatives in the post feeding antibiotic era. *Med Res Rev*, 39(3): 831–859.

https://doi.org/10.1002/med.21542

44. Rai A, Ferrão R, Palma P, *et al*., 2022, Antimicrobial peptide-based materials: Opportunities and challenges. *J Mater Chem B*, 10(14): 2384–2429.

https://doi.org/10.1039/D1TB02617H

45. Zi Y, Yang K, He J, *et al*., 2022, Strategies to enhance drug delivery to solid tumors by harnessing the EPR effects and alternative targeting mechanisms. *Adv Drug Deliv Rev*, 188: 114449.

https://doi.org/10.1016/j.addr.2022.114449

46. Zocchi ML, Vindigni V, Pagani A, *et al*., 2019, Regulatory, ethical, and technical considerations on regenerative technologies and adipose-derived mesenchymal stem cells. *Eur J Plastic Surg*, 42: 531–548.

https://doi.org/10.1007/s00238-019-01571-5

47. Khan A, Mann L, Papanna R, *et al*., 2017, Mesenchymal stem cells internalize Mycobacterium tuberculosis through scavenger receptors and restrict bacterial growth through autophagy. *Sci Rep*, 7(1): 15010.

https://doi.org/10.1038/s41598-017-15290-z

48. Doi K, Leelahavanichkul A, Yuen PS, *et al*., 2009, Animal models of sepsis and sepsis-induced kidney injury. *J Clin Investig*, 119(10): 2868–2878.

https://doi.org/10.1172/JCI39421

49. Krasnodembskaya A, Song Y, Fang X, *et al*., 2010, Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells*, 28(12): 2229–2238.

https://doi.org/10.1002/stem.544

50. Ding J, Maxwell A, Adzibolosu N, *et al*., 2022, Mechanisms of immune regulation by the placenta: Role of type I interferon and interferon-stimulated genes signaling during pregnancy. *Immunol Rev*, 308(1): 9–24.

https://doi.org/10.1111/imr.13077

51. Yao X, Wei W, Wang X, *et al*., 2019, Stem cell derived exosomes: MicroRNA therapy for age-related musculoskeletal disorders. *Biomaterials*, 224: 119492.

https://doi.org/10.1016/j.biomaterials.2019.119492

52. Kurtz A., 2008, Mesenchymal stem cell delivery routes and fate. *Int J Stem Cells*, 1(1): 1–7.

https://doi.org/10.15283/ijsc.2008.1.1.1

53. Herrmann IK, Wood MJ, Fuhrmann G, 2021, Extracellular vesicles as a next-generation drug delivery platform. *Nat Nanotechnol*, 16(7): 748–759.

https://doi.org/10.1038/s41565-021-00931-2

54. Dunn CM, Kameishi S, Grainger DW, *et a*., 2021, Strategies to address mesenchymal stem/stromal cell heterogeneity in immunomodulatory profiles to improve cell-based therapies. *Acta Biomater*, 133: 114–125.

https://doi.org/10.1016/j.actbio.2021.03.069

55. Zhang T, Lin R, Wu H, *et al*., 2022, Mesenchymal stem cells: A living carrier for active tumor-targeted delivery. *Adv Drug Deliv Rev*, 185: 114300.

https://doi.org/10.1016/j.addr.2022.114300

56. Teixeira MC, Carbone C, Sousa MC, *et al*., 2020, Nanomedicines for the delivery of antimicrobial peptides (AMPs). *Nanomaterials* (*Basel*), 10(3): 560.

https://doi.org/10.3390/nano10030560

57. Gao T, Huang F, Wang W, *et al*., 2022, Interleukin-10 genetically modified clinical-grade mesenchymal stromal cells markedly reinforced functional recovery after spinal cord injury via directing alternative activation of macrophages. *Cell Mole Biol Lett*, 27(1): 27.

https://doi.org/10.1186/s11658-022-00325-9

58. Chen CH, Bepler T, Pepper K, *et al*., 2022, Synthetic molecular evolution of antimicrobial peptides. *Curr Opin Biotechnol*, 75: 102718.

59. Zhang J, Zhang W, Sun T, *et al*., 2022, The influence of intervertebral disc microenvironment on the biological

behavior of engrafted mesenchymal stem cells. *Stem Cells Int*, 2022: 8671482.

https://doi.org/10.1155/2022/8671482

60. Cheng Y, Cao X, Qin L, 2020, Mesenchymal stem cell-derived extracellular vesicles: A novel cell-free therapy for sepsis. *Front Immunol*, 11: 647.

https://doi.org/10.3389/fimmu.2020.00647

61. Guastaldi RB, Secoli SR, 2011, Drug interactions of anti-microbial agents used in hematopoietic stem cell transplantation. *Rev Lat Am Enfermagem*, 19: 960–967.

https://doi.org/10.1590/s0104-11692011000400015

62. Lee AS, Tang C, Rao MS, *et al*., 2013, Tumorigenicity as a clinical hurdle for pluripotent stem cell therapies. *Nat Med*, 19(8): 998–1004.

https://doi.org/10.1038/nm.3267

63. Lukomskyj AO, Rao N, Yan L, *et al*., 2022, Stem cell-based tissue engineering for the treatment of burn wounds: A systematic review of preclinical studies. *Stem Cell Rev Rep*, 18(6): 1926–1955.

https://doi.org/10.1007/s12015-022-10341-z

64. Bolli R, Hare JM, March KL, *et al*., 2018, Rationale and design of the CONCERT-HF trial (combination of mesenchymal and c-kit+ cardiac stem cells as regenerative therapy for heart failure). *Circ Res*, 122(12): 1703–1715.

https://doi.org/10.1161/CIRCRESAHA.118.312978

65. Hanson SE, Bentz ML, Hematti P, 2010, Mesenchymal stem cell therapy for nonhealing cutaneous wounds. *Plast Reconstruct Surg*, 125(2): 510–516.

https://doi.org/10.1097/PRS.0b013e3181c722bb

66. Mousaei Ghasroldasht M, Seok J, Park HS, *et al*., 2022, Stem cell therapy: From idea to clinical practice. *Int J Mole Sci*, 23(5): 2850.

https://doi.org/10.3390/ijms23052850

67. Harapanhalli RS, 2010, Food and drug administration requirements for testing and approval of new radiopharmaceuticals. *Semin Nucl Med*, 40(5): 364–384.

https://doi.org/10.1053/j.semnuclmed.2010.05.002

68. Zetterberg H, Bendlin BB, 2021, Biomarkers for Alzheimer's disease-preparing for a new era of disease-modifying therapies. *Mol Psychiatry*, 26(1): 296–308.

https://doi.org/10.1038/s41380-020-0721-9

69. Kanie K, Sakai T, Imai Y, *et al*., 2019, Effect of mechanical vibration stress in cell culture on human induced pluripotent stem cells. *Regen Ther*, 12: 27–35.

https://doi.org/10.1016/j.reth.2019.05.002

70. Thirumala S, Goebel WS, Woods EJ, 2009, Clinical grade adult stem cell banking. *Organogenesis*, 5(3): 143–154.

https://doi.org/10.4161/org.5.3.9811

71. Le Blanc K, Davies LC, 2015, Mesenchymal stromal cells and the innate immune response. *Immunol Lett*, 168(2): 140–146.

https://doi.org/10.1016/j.imlet.2015.05.004

72. Zhao Y, Pu M, Zhang J, *et al*., 2021, Recent advancements of nanomaterial-based therapeutic strategies toward sepsis: Bacterial eradication, anti-inflammation, and immunomodulation. *Nanoscale*, 13(24): 10726–10747.

https://doi.org/10.1039/D1NR02706A

73. Qin H, Zhao A, 2020, Mesenchymal stem cell therapy for acute respiratory distress syndrome: From basic to clinics. *Protein Cell*, 11(10): 707–722.

https://doi.org/10.1007/s13238-020-00738-2

74. Lafaille FG, Harschnitz O, Lee YS, *et al*., 2019., Human SNORA31 variations impair cortical neuron-intrinsic immunity to HSV-1 and underlie herpes simplex encephalitis. *Nat Med*, 25(12): 1873–1884.

https://doi.org/10.1038/s41591-019-0672-3

75. Shi C, Zhu Y, Ran X, *et al*., 2006, Therapeutic potential of chitosan and its derivatives in regenerative medicine. *J Surg Res*, 133(2): 185–192.

https://doi.org/10.1016/j.jss.2005.12.013

76. Malandrakis AA, Kavroulakis N, Chrysikopoulos CV, 2022, Metal nanoparticles against fungicide resistance: Alternatives or partners? *Pest Manag Sci*, 78(10): 3953–3956.

https://doi.org/10.1002/ps.7014

77. Melkoumian Z, Weber JL, Weber DM, *et al*., 2010, Synthetic peptide-acrylate surfaces for long-term self-renewal and cardiomyocyte differentiation of human embryonic stem cells. *Nat Biotechnol*, 28(6): 606–610.

https://doi.org/10.1038/nbt.1629

78. Shepherd FR, McLaren JE, 2020, T cell immunity to bacterial pathogens: Mechanisms of immune control and bacterial evasion. *Int J Mole Sci*, 21(17): 6144.

https://doi.org/10.3390/ijms21176144

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Gene & Protein in Disease

REVIEW ARTICLE

The roles and mechanisms of ETS1 in autoimmune diseases and cancers: A comprehensive review

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Abstract

E26 transformation-specific-1 (ETS1) is a founding member of the ETS transcription factor family. This transcription factor is highly conserved in amino acid sequences and highly expressed in many immune tissues, such as the thymus, spleen, and lymph gland. ETS1 plays multiple regulatory roles in immune-related diseases and acts as a transcriptional activator or inhibitor of many genes to regulate immune cell differentiation, development, apoptosis, and tumor occurrence. The expression level of ETS1 is correlated with disease severity. However, the molecular mechanisms behind disease and tumor progression mediated by ETS1 have not been fully elucidated. In addition, the therapeutic potential of ETS1 in clinical treatment remains to be further explored. Here, we review and summarize the molecular structure and functions of ETS1 and then focus on the roles of ETS1 in the occurrence of autoimmune diseases and cancers. This review provides a reference corroborating ETS1 as a potential therapeutic target for immune-related diseases and cancers.

Keywords: ETS1; Autoimmune diseases; Cancer; Immunity

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1. Introduction

E26 transformation-specific-1 (ETS1) is a member of the ETS transcription factor family and has a unique E26 transformation-specific (ETS) domain (DNA binding domain, DBD) composed of 85 amino acids. In humans, the ETS family members are encoded by 28 genes and play regulatory roles in cell survival, growth, proliferation, differentiation, angiogenesis, apoptosis, metastasis, and metabolisms[1,2]. Similar to EST1, ETS2 is also one of the founding members of the ETS family and regulates transcription by binding to ETS sequences. ETS1 shares a close resemblance to ETS2, in terms of their amino acid and structural domains, indicating that they have nearly identical DNA-binding domains[2,3]. Friend leukemia integration 1 (*FLI1*) — another member of the ETS family — is located at close proximity to linked to *ETS1* on the chromosome, and both genes are arranged in a head-to-head fashion[2,4]. Compared with other members of the ETS

transcription factor family, the ETS1 protein not only is highly conserved in its ETS domain but also exhibits extensive conservation throughout its entire protein sequence, indicating that the amino acid sequence of ETS1 as a whole is essentially under evolutionary pressure and that each component may be crucial for the growth and differentiation of metazoans.

Due to the high conservation and high expression of ETS1 in immune tissues, the mechanisms and its effects on the occurrence of immune-related diseases have attracted increasing attention. An in-depth study showed that the ETS1 protein plays pivotal roles in stem cell development, cell aging, and death, as well as tumor progression by acting as a transcriptional activator or inhibitor of several genes[2]. The roles of ETS1 are also tightly connected to immune-related diseases[5]. This paper reviews the structure and function of ETS1 as well as its roles and mechanisms in the occurrence of immune-related diseases.

2. Structure and function of ETS1

The ETS1 protein is a nuclear protein, with a size of 54 kDa that mainly functions as a transcription activator, but other studies have shown that it can also inhibit gene transcription[6]. The human ETS1 protein is composed of 441 amino acids, which are composed of a pointed domain (54 – 134 aa), a transactivation domain (TAD; 135 – 242 aa), an exon VII domain (242 – 331 aa), an ETS domain (331 – 415 aa), and an autoinhibitory module (two separated sequences, one in the exon VII domain between 301 aa and 330 aa and the other at the C-terminal end between 416 and 441 aa) (Figure 1). These structural domains are adjacent to each other on the chromosome and play different regulatory roles.

The pointed domain, a sterile alpha motif domain, serves as a platform for protein-protein interactions. In addition, extracellular regulated protein kinase 2 (ERK2) can partially bind to this region and phosphorylate ETS1 at Thr38 and Ser41[7]. The exon VII domain contains an inhibitory domain that is an autoinhibitory unit. Full-length ETS1 (fl-ETS1) and DeltaVII-ETS1 are the two naturally occurring protein isoforms produced by alternative splicing of exon VII[8]. The loss of the inhibitory domain in the exon VII domain causes the elimination of the autoinhibitory module in DeltaVII-ETS1, which in turn unleashes the complete DNA binding activity of ETS1[9]. Afterward, the ETS1 protein can perform better DNA binding, achieving gene expression and transcriptional activity superior to those of the fl-ETS1 protein. However, the balance in differential regulation between DeltaVII-ETS1 and fl-ETS1 suggests a possible mechanism that governs lymphoid maturation and homeostasis[8]. The ETS domain possesses a winged helix turn helix (wHTH) structure with three α-helices and four β-sheets that exclusively identify and attach to a core purine-rich 5′-GGAA/T-3′ motif[10]. The recognition mechanism of ETS1 through homologous sequences in the ETS domain has also been described[11]. The autoinhibitory module consists of two inhibitory domains adjacent to the ETS domain, which prevents ETS1 from binding to DNA. Two inhibitory domains constitute four α-spiral structures: the HI-1 and HI-2 helices are located in the N-terminal region, while the H4 and H5 helices are in the C-terminal region[12,13]. These helices form a spiral bundle that interacts with the ETS domain, thus inhibiting the binding of the ETS domain to DNA[14].

3. Effect and mechanism of action of ETS1 in the occurrence of autoimmune diseases

3.1. Systemic lupus erythematosus (SLE)

SLE is an autoimmune disease that interferes with the immune response, damaging multiple organs serving the host immune system. Two previous genome-wide association studies conducted in Han Chinese and Asian populations have shown that susceptibility to SLE is correlated with *ETS1* gene mutations[15,16]. A recent research, which utilized single-cell RNA sequencing to analyze peripheral blood mononuclear cells of multiple SLE age groups, has shown that *ETS1* is a susceptibility gene associated with SLE[17]. These results all indicate a close relationship between ETS1 and SLE. Moreover, a study has shown that ETS1 is a critical negative regulatory factor in Th17 cells[18]. Recently, investigations focusing on whether the negative regulatory effect of ETS1 on B cells

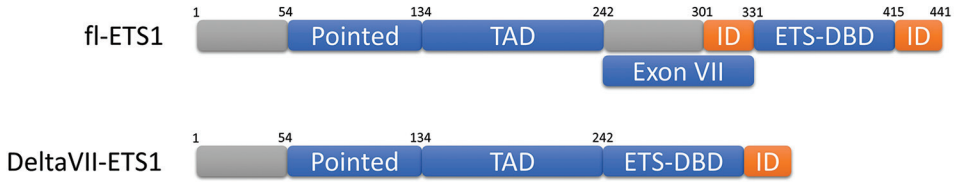

Figure 1. Schematic structure of the ETS1 protein. The schematic shows the positions of the different domains within the fl-ETS1 and DeltaVII-ETS1 proteins. The two inhibitory domains (IDs) flanking the ETS-DBD (DNA binding domain) form an autoinhibitory module that controls ETS1 DNA binding activity. DeltaVII-ETS1 lacks part of the inhibitory module and forms more stable complexes with DNA.

and Th17 cells are directly linked to the occurrence and development of SLE has been gaining traction, but the specific mechanism involved has yet to be determined[18-23].

Moreover, a recent article by Kim *et al.* suggested that *ETS1* inhibits T follicular helper Type 2 (Tfh2) cells, thereby preventing the onset of SLE[24]. Specifically, a decrease in ETS1 expression in $CD4^+$ T cells in SLE patients leads to an increase in the secretion of IL-4 by $GATA\text{-}3^+Bcl6^+$ Tfh2 cells, thereby increasing the production of the autoantibodies IgE and IgG1[24]. Kim *et al.* also demonstrated that SLE autoimmunity was caused by the deletion of *ETS1* specifically in $CD4^+$ T cells, rather than in B cells or dendritic cells (DCs), and was linked to the spontaneous expansion of Tfh2 cells[24]. This research provides evidence that ETS1 regulates $CD4^+$ T-cell differentiation in the pathogenesis of SLE.

It has been shown that lacking ETS1 leads to more intrinsic changes in B cells, including an expansion of the B cell population in the marginal zone, increased antibody secretion in plasma cells, an increased B-cell proportion with a memory phenotype, and an increased autoantibody titer[25]. Moreover, these changes are not accompanied by significant T-cell over activation. However, changes in biological processes that are regulated by ETS1, including the development of antibody-secreting cells and memory B cells and the production of autoantibodies, are not obvious in B-cell *ETS1*-knockout mice compared with those in mice with complete *ETS1* knockout[25]. A recent study revealed that ETS1 downregulation could increase miR-326 expression in B cells of SLE patients, thereby driving the growth of plasmablasts and the generation of antibodies[26]. Although the mechanism through which ETS1 participates in SLE still lacks clarity, it is obvious that B cells play an important role in this regard. This is evidenced by the role of abnormal interactions between T cells and B cells induced by ETS1 knockdown in the mechanism underlying SLE pathogenesis, although SLE-like symptoms are manifested in patients with a lack of ETS1 in T cells, rather than in B cells[24].

3.2. Rheumatoid arthritis (RA)

RA is a complex autoimmune disease that primarily affects joints. In RA, the immune system attacks the host joint tissues, causing pain and swelling, and impairing joint function. However, the pathogenesis of RA is yet unknown. It has been found that the single nucleotide polymorphism rs73013527 of *ETS1* was significantly positively correlated with RA-sensitive DAS28 and C-reactive protein in the Han Chinese population[27]. At present, studies on the function and mechanism of ETS1 in the pathogenesis of RA are scarce. However, through a series of univariate and multivariate studies, researchers found that *ETS1* rs73013527 has an indirect impact on serum receptor activator of nuclear factor kappa B ligand (RANKL) levels in RA patients[28]. A recent study also established ETS1 as a crucial transcription factor by virtue of its ability to promote the production of RANKL and tissue-destructing fibroblasts[29]. In addition, ETS1 may affect serum RANKL levels through the cytokine network, especially given that RA patients have markedly increased IL-17 levels[28].

3.3. Autoimmune thyroiditis (AIT)

AIT is a disease in which lymphocytes infiltrate the impaired thyroid through antibodies. Recent studies have shown that high miR-326 expression is associated with a significant increase of Th17 cells and decreased ETS1 protein levels for Th17 differentiation during the onset and development of AIT[30,31]. miR-326 may target the Ets1 protein to trigger iodide-induced AIT since the expression of Ets1 in AIT mice is adversely linked with miR-326 levels[30]. A subsequent study further verified that miR-326 levels lead to AIT by controlling the Th17/Treg balance through ETS1[31].

3.4. Multiple sclerosis (MS)

MS, an autoimmune disease, is a persistent neurological condition that primarily impacts the central nervous system, encompassing both the brain and the spinal cord. Li *et al.* reported that the upregulation of *miR-1-3p* in patients with recurrent MS and Th17 cells, as well as *miR-1-3p* expression, was strongly linked with the severity of MS. In the peripheral blood mononuclear cells of relapsed patients with recurrent MS, the expression of *ETS1* (a target gene of *miR-1-3p*) was reduced, while the upregulation of ETS1 inhibited the expression of inflammation-related genes (*CXCL3*, *CSF2,* and *IL-23R*), thereby decreasing the pathogenicity of Th17 cells[32]. The overexpression of *miR-1-3p* in naïve $CD4^+$ T cells has a positive effect on Th17 cell differentiation and is positively correlated with the progression of MS. Targeting miR-1-3p with ETS1 could reduce the level of miR-1-3p and inhibit the pathogenic response of Th17 cells[32]. Dysfunction of the blood-brain barrier (BBB) has been recognized as a contributor to MS, and disturbances in vascular stability and barrier function are caused by the endothelial-to-mesenchymal transition (EndMT), a mechanism linked to endothelial dysfunction. The effect of EndMT in MS is still unclear, but recently, a study found that ETS1 serves as the primary regulator of EndMT, which is connected to diminished barrier integrity[33], providing compelling evidence on the pivotal functional role of EndMT in the disruption of the blood-brain barrier, which is a critical feature in MS pathogenesis. Moreover, the authors proposed that ETS1 might serve as

a key molecular switch for EndMT to target the onset of MS[33].

4. Effect and mechanisms of ETS1 in cancers

4.1. Expression of ETS1 in cancers

Current research shows that *ETS1* is highly expressed in various types of cancer cells, such as colorectal cancer, breast cancer, and endometrial cancer cells, and is related to poor differentiation, high potential invasion, high angiogenesis activity, and increased risk of lymph node transition[9]. However, the specific molecular mechanism by which ETS1 operates in the context of cancer remains unclear, pending further in-depth exploration and comprehensive elucidation.

Several investigations have shown that ETS1 promotes the development of breast cancer, although the molecular mechanism underlying this finding remains obscure[34-36]. In addition, a recent study indicated that inducing ferroptosis in breast cancer through ETS1 degradation facilitates the promotion of the ETS1-SYVN1 interaction by sculponeatin A[37]. Breast cancer cells can promote endothelial cell morphogenesis in a paracrine manner and physically connect with endothelial cells since ETS1 is a critical regulator of their angiogenic potential[38]. Furthermore, differential regulation of ETS1 isoforms (fl-ETS1 and DeltaVII-ETS1) can affect lymphoid maturation and homeostasis, and these isoforms exhibit significant differences in DNA binding, transcriptional target specificity, and protein-protein interactions[8]. In cancer, the balance between fl-ETS1 and DeltaVII-ETS1 is disrupted. For example, it has been found that a big majority of MDA-MB-231 breast cancer cells have a high expression of fl-ETS1, and only 10% of these cells exhibit concomitant expression of DeltaVII-ETS1[39]. However, in some cases, the high expression of ETS1 may have an inhibitory effect on breast cancer. Furlan *et al.* revealed that ETS1 not only promotes malignant tumors by endowing them with invasive features but also weakens the growth of breast tumor cells, thus presenting contrasting capabilities to promote metastasis and to inhibit the growth of primary tumors[40]. Recently, Kim *et al.* reported that ETS1 not only directly activates downstream targets related to tumor cell proliferation and growth, but also activates additional tumor suppressor genes, such as *ADAMTS9*, *TXNIP*, *STAT5A*, and *NOTCH1*, through transactivation[41].

4.2. ETS1 and matrix metalloproteinases in cancer

The specific way in which the highly expressed *ETS1* gene promotes the development of cancer cells has not been elucidated, but a large number of studies have shown that ETS1, along with other ETS proteins, facilitates the potential invasion of cancer cells by directing the overexpression of matrix metalloproteinases (MMPs). Nazir *et al.* demonstrated that the transfection of breast cancer cell lines with *ETS1* siRNA resulted in notable decreases in both ETS1 and MMP-9. In addition, the knockdown of *ETS1* not only attenuated the invasive potential of cancer cells but also induced alterations in the expression of epithelial-mesenchymal transition (EMT) markers[42].

The correlation between ETS1 and MMP synthesis has also been confirmed in a range of cancer types, including pancreatic cancer, colon cancer prostate cancer, and ovarian cancer cells. Multiple signals converge into ETS1 to increase the production of MMP. Within pancreatic cancer cells, the presence of prostaglandin E2 triggers ETS1-driven upregulation of MMP2 expression[43]. In colon cancer cells, the activation of β6 integrin enhances the ETS1-mediated expression of both MMP3 and MMP9[44]. In ovarian cancer cells, glycogen synthase kinase-3β (GSK3β) mediates ETS1 phosphorylation, promotes the progression of ovarian cancer, induces transcriptional activation of MMP9, and increases cell migration. GSK3β inhibitors have been demonstrated *in vivo* to inhibit endogenous ETS1 expression and induce MMP9 expression[45].

4.3. ETS1 and phosphorylation in cancers

The Ras/Raf/MEK/ERK1/2 pathway plays a crucial role in modulating ETS1 activity at the posttranslational level[46]. The activation of these pathways can lead to N-terminal phosphorylation of the ETS1 protein at threonine 38 and super activation of ETS1[47]. The expression of ETS1 in the biopsy samples of human breast cancer is correlated with the level of phosphorylated Raf, suggesting that the over activation of ETS1 contributes to the progression of breast cancer[48]. This association underscores the potential significance of ETS1 in the context of breast cancer pathogenesis.

The poor prognosis of human ovarian cancer is possibly accounted for by the enhanced expression of ETS1. Tomar *et al.* made a significant discovery that ETS1 acts as a vital transcription factor triggered in ovarian cancer cells by the surrounding microenvironment and facilitates metastatic colonization by orchestrating the transcriptional upregulation of its target FAK[49]. The serotonin-rich region (SRR) in ETS1 can automatically inhibit its ability to bind DNA. The ability of SRR to inhibit inflammation has been found to be significantly improved by phosphorylation[50]. However, the molecular processes that control the regulation of ETS1 structure and activity by phosphorylation remain to be elucidated. Nevertheless, a study by Ning *et al.* has elucidated the reasons for the

phosphorylation stability of the ETS1 core in the SRR region[50].

4.4. ETS1 and microRNAs in cancers

Recently, increasing evidence has suggested that microRNAs (miRNAs) are important regulators of ETS1 expression. It has been proven that more than ten different miRNAs can directly target the 3'-UTR of human *ETS1* RNA to downregulate its expression. Moreover, the binding sites for these miRNAs are highly clustered within the *ETS1* 3'-UTR[9] (Figure 2). Previous studies have shown that miR-1, miR-129-5p, miR-193b, and miR-499 can downregulate ETS1 expression in human liver cancer cells, subsequently inhibiting their ability to migrate and invade[51-54]. *ETS1* can be targeted by miR-9 and miR-145 in gastric cancer cells[55,56]. Targeting of *ETS1* by miR-377 in clear cell renal cell carcinoma (ccRCC) cells can decrease the ETS1 expression and reduce the ability of ccRCC cells to proliferate, migrate, and invade, while targeting of *ETS1* by miR-124 and miR-125b can decrease ETS1 expression and suppress the progression of breast cancer[57-59]. A growing understanding toward the regulatory effects of these miRNAs has led to the recent discovery of several new miRNAs target *ETS1*.

MicroRNAs play a role in promoting or inhibiting tumor development, depending on the cellular background. Hua *et al.* conducted a thorough investigation into the regulatory effects of miR-139-5p on aerobic glycolysis in hepatocellular carcinoma (HCC). Their study, which included clinical samples and mouse models, revealed the significant regulatory interplay between miR-139-5p and *ETS1*, which modulates not only aerobic glycolysis but also proliferation and metastasis of HCC cells[60]. miR-532-5p is among the miRNAs that are significantly downregulated in renal cell carcinoma (RCC) cells. Zhai *et al.* reported an intriguing mechanism by which miR-532-5p abrogates the proliferation of RCC cells through disruption of the ETS1-mediated positive feedback loop through the KRAS-NAP1L1/P-ERK axis, revealing a crucial regulatory pathway involved in RCC tumorigenesis[61].

Long non-coding RNAs (lncRNAs) have been shown to regulate the development of cancer in numerous studies. Recently, Jin *et al.* reported that ETS1 promotes the transcription of *SNHG10,* which plays a pivotal role in the malignant behavior of glioma cells through the intricate SNHG10/miR-532-3p/FBXL19 signaling pathway, highlighting the multifaceted regulatory mechanisms involved in glioma progression[62]. Due to the increase in global incidence rate, ccRCC has become the predominant type of RCC in the adult population[63]. Microarray-based expression analysis revealed that *SBF2-AS1* was differentially expressed in ccRCC, and the downregulation of *SBF2-AS1* could be achieved by inhibiting miR-338-3p, which targets *ETS1*, a tumor inhibitor in ccRCC[64]. The ETS1-mediated lncRNA muskelin 1 antisense RNA (MKLN1-AS) has been shown to enhance the malignant phenotypes of HCC cells[65]. Recently, Sun *et al.* reported that the long intergenic noncoding RNA 01016 (LINC01016) is key to the development of breast cancer[66].

4.5. ETS1 and the telomerase reverse transcriptase (*TRET*) promoter in cancers

Cellular immortalization is an indispensable step in cancer development and is achieved through the sustenance of the expression of telomerase, which grants the cancer cells infinite capability to divide[67]. *TRET* is the catalytic component of telomerase, which remains at low expression levels in normal somatic cells but is re-expressed in cancers[68,69]. Mutation of the *TRET* promoter is associated with increased TRET expression in melanoma, and ETS1 is significant for TRET upregulation[67,69]. A previous study indicated that the binding of ETS alone to the mutated *TRET* promoter (C250T) is insufficient to drive TRET reactivation, as a member of the NF-κB family of transcription factors (p52) that acts downstream of noncanonical NF-κB signaling is required for interaction[68]. p52 interacts with ETS1 to form a heterotetramer that can counteract autoinhibition of ETS1, and the p52/ETS1 heterotetramer can drive reactivation of the mutated *TRET* promoter (C146T) in multiple cancer types[70]. Moreover, Vallarelli *et al.* suggested that ETS1 is phosphorylated in

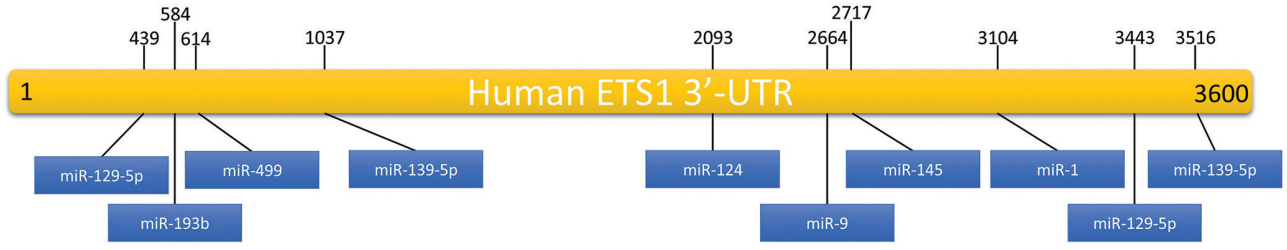

Figure 2. Regulation of the 3′-UTR of human *ETS1* mRNA by microRNAs. Only miRNA binding sites that have been verified by mutational analysis are shown. miRNA binding sites are clustered, instead of being randomly distributed, within the *ETS1* 3′-UTR.

an ERK-dependent manner in all melanoma cell lines, that mutations in *TRET* create ETS transcription factor-binding sites, and that ETS1 binding to the mutated *TRET* promoter leads to re-expression of TRET[67]. Furthermore, Gabler *et al.* reported that the coexistence of mutations in serine/threonine kinase ($BRAF^{V600E}$) and *TRET* promoter leads to cancer cell proliferation and immortalization, and ETS1 functionally links these two driver alterations[71]. These studies provide significant insights into the roles of ETS1 in regulating the immortalization of cancer cells by the mutated *TRET* promoter, which could contribute to immense therapeutic implications.

4.6. The role of ETS1 in B-cell and T-cell malignancies

ETS1 has crucial roles in the development of lymphoid tissue and the activation of lymphocytes, indicating that it has a potential relationship with B-cell and T-cell malignancies. A previous study focused on the multiple roles and regulatory mechanisms of ETS1 in hematological development, including T-cell and natural killer (NK) cell activation and B-cell maturation and differentiation[72,73]. With in-depth research on the mechanisms of action of ETS1 in diseases, its role as a key transcription factor in B-cell and T-cell malignancies has gradually been revealed. In classical Hodgkin's lymphoma (cHL), *ETS1* is hypermethylated exclusively and is markedly decreased in the cHL cell line. Recurrent deletions and loss of ETS1 expression could contribute to the potential escape and survival of Hodgkin and Reed-Sternberg cells (HRS) and impair B-cell development[74]. In diffuse large B-cell lymphoma (DLBCL), 11q24.3 genomic lesions are correlated with high expression levels of *ETS1* and *FLI1*, and overexpression of these two genes could contribute to the pathogenesis of DLBCL in a cooperative manner by deregulating genes involved in the germinal center expression program and cell proliferation[75]. Further study of DLBCL demonstrated that *ETS1* silencing affected genes involved in B-cell signaling, differentiation, cell cycle, and immune processes, highlighting its role in lymphomagenesis, particularly in activated-like B-cell (ABC) DLBCL[73]. Recent research has indicated that B-cell receptor-mediated ETS1 phosphorylation at threonine 38 is important for the growth of DLBCL cells and is related to the ABC-DLBCL phenotype but is predictive of poor outcome in patients with germinal center B-cell-like (GCB) DLBCL[76]. Drug-mediated inhibition of ETS1 phosphorylation could have a positive effect on lymphoma patients[76]. Regarding the role of ETS1 in T-cell malignancies, a recent study showed that Notch activation is closely related to the induction of T-cell acute lymphoblastic leukemia[77]. Moreover, Luchtel *et al.* reported that the expression of ETS1 was significantly increased in adult T-cell leukemia/lymphoma of North American – descendent patient (NA-ATLL) cell lines and primary tumor samples, and knocking down *ETS1* in NA-ATLL cells resulted in cell growth inhibition, indicating that ETS1 is a new dominant oncogenic transcription regulator in NA-ATLL[78].

5. Conclusion

This comprehensive review provides an in-depth examination of the outcomes and functions associated with ETS1. The myriad roles of this transcription factor assume in immune-related diseases that are reviewed. Furthermore, this review explores the diverse contributions of ETS1 to the onset and progression of various diseases, incorporating the most recent findings from scientific research. ETS1 not only induces immune-related diseases but also correlates with disease severity. Although these studies provide us with a clear understanding of the role of *ETS1* in immune-related diseases, additional specific molecular mechanisms have not been elucidated. We believe that the continuous development of research methods and technologies can enable further exploration of the diverse and complex roles of ETS1 in immune-related diseases, which may have significant implications for clinical treatment.

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Conflict of interest

The authors have no personal, financial, or institutional interest with regard to any of the drugs, materials, or devices described in this article.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

Not applicable.

References

1. Wang Y, Huang Z, Sun M, *et al*., 2023, ETS transcription factors: Multifaceted players from cancer progression to tumor immunity. *Biochim Biophys Acta Rev Cancer*, 1878(3): 188872.

   https://doi.org/10.1016/j.bbcan.2023.188872

2. Garrett-Sinha LA, 2013, Review of Ets1 structure, function, and roles in immunity. *Cell Mol Life Sci*, 70(18): 3375–3390.

   https://doi.org/10.1007/s00018-012-1243-7

3. Fry EA, Mallakin A, Inoue K, 2018, Translocations involving ETS family proteins in human cancer. *Integr Cancer Sci Ther*, 5(4): 10.15761/ICST.1000281.

   https://doi.org/10.15761/ICST.1000281

4. He YS, Yang XK, Hu YQ, *et al*., 2021, Emerging role of Fli1 in autoimmune diseases. *Int Immunopharmacol*, 90: 107127.

   https://doi.org/10.1016/j.intimp.2020.107127

5. Garrett-Sinha LA, 2023, An update on the roles of transcription factor Ets1 in autoimmune diseases. *WIREs Mech Dis*, 15(6): e1627.

   https://doi.org/10.1002/wsbm.1627

6. Wang C, Kam RK, Shi W, *et al*., 2015, The Proto-oncogene transcription factor Ets1 regulates neural crest development through histone deacetylase 1 to mediate output of bone morphogenetic protein signaling. *J Biol Chem*, 290(36): 21925–21938.

   https://doi.org/10.1074/jbc.M115.644864

7. Piserchio A, Warthaka M, Kaoud TS, *et al*., 2017, Local destabilization, rigid body, and fuzzy docking facilitate the phosphorylation of the transcription factor Ets-1 by the mitogen-activated protein kinase ERK2. *Proc Natl Acad Sci U S A*, 114(31): E6287–E6296.

   https://doi.org/10.1073/pnas.1702973114

8. Higuchi T, Bartel FO, Masuya M, *et al*., 2007, Thymomegaly, microsplenia, and defective homeostatic proliferation of peripheral lymphocytes in p51-Ets1 isoform-specific null mice. *Mol Cell Biol*, 27(9): 3353–3366.

   https://doi.org/10.1128/MCB.01871-06

9. Dittmer J, 2015, The role of the transcription factor Ets1 in carcinoma. *Semin Cancer Biol*, 35: 20–38.

   https://doi.org/10.1016/j.semcancer.2015.09.010

10. Sizemore GM, Pitarresi JR, Balakrishnan S, *et al*., 2017, The ETS family of oncogenic transcription factors in solid tumours. *Nat Rev Cancer*, 17(6): 337–351.

    https://doi.org/10.1038/nrc.2017.20

11. Huang K, Xhani S, Albrecht AV, *et al*., 2019, Mechanism of cognate sequence discrimination by the ETS-family transcription factor ETS-1. *J Biol Chem*, 294(25): 9666–9978.

    https://doi.org/10.1074/jbc.RA119.007866

12. Skalicky JJ, Donaldson LW, Petersen JM, *et al*., 1996, Structural coupling of the inhibitory regions flanking the ETS domain of murine Ets-1. *Protein Sci*, 5(2): 296–309.

    https://doi.org/10.1002/pro.5560050214

13. Donaldson LW, Petersen JM, Graves BJ, *et al*., 1996, Solution structure of the ETS domain from murine Ets-1: a winged helix-turn-helix DNA binding motif. *EMBO J*, 15(1): 125–134.

    https://doi.org/10.1002/j.1460-2075.1996.tb00340.x

14. Karolak A, van der Vaart A, 2012, Importance of local interactions for the stability of inhibitory helix 1 in apo Ets-1. *Biophys Chem*, 165–166: 74–78.

    https://doi.org/10.1016/j.bpc.2012.03.007

15. Han JW, Zheng HF, Cui Y, *et al*., 2009, Genome-wide association study in a Chinese Han population identifies nine new susceptibility loci for systemic lupus erythematosus. *Nat Genet*, 41(11): 1234–1237.

    https://doi.org/10.1038/ng.472

16. Yang W, Shen N, Ye DQ, *et al*., 2010, Genome-wide association study in Asian populations identifies variants in ETS1 and WDFY4 associated with systemic lupus erythematosus. *PLoS Genet*, 6(2): e1000841.

    https://doi.org/10.1371/journal.pgen.1000841

17. Zhong J, Ding R, Jiang H, *et al*., 2022, Single-cell RNA sequencing reveals the molecular features of peripheral blood immune cells in children, adults and centenarians. *Front Immunol*, 13: 1081889.

    https://doi.org/10.3389/fimmu.2022.1081889

18. Zhang J, Zhang Y, Zhang L, *et al*., 2013, Epistatic interaction between genetic variants in susceptibility gene ETS1 correlates with IL-17 levels in SLE patients. *Ann Hum Genet*, 77(4): 344–350.

    https://doi.org/10.1111/ahg.12018

19. John SA, Clements JL, Russell LM, *et al*., 2008, Ets-1 regulates plasma cell differentiation by interfering with the activity of the transcription factor Blimp-1. *J Biol Chem*, 283(2): 951–962.

    https://doi.org/10.1074/jbc.M705262200

20. Jin L, Fang X, Dai C, *et al*., 2019, The potential role of Ets-1

and miR-326 in CD19(+)B cells in the pathogenesis of patients with systemic lupus erythematosus. *Clin Rheumatol*, 38(4): 1031–1038.

https://doi.org/10.1007/s10067-018-4371-0

21. Moisan J, Grenningloh R, Bettelli E, *et al*., 2007, Ets-1 is a negative regulator of Th17 differentiation. *J Exp Med*, 204(12): 2825–2835.

https://doi.org/10.1084/jem.20070994

22. Na SY, Park MJ, Park S, *et al*., 2016, MicroRNA-155 regulates the Th17 immune response by targeting Ets-1 in Behcet's disease. *Clin Exp Rheumatol*, 34(6 Suppl 102): S56–S63.

23. Luo W, Mayeux J, Gutierrez T, *et al*., 2014, A balance between B cell receptor and inhibitory receptor signaling controls plasma cell differentiation by maintaining optimal Ets1 levels. *J Immunol*, 193(2): 909–920.

https://doi.org/10.4049/jimmunol.1400666

24. Kim CJ, Lee CG, Jung JY, *et al*., 2018, The transcription factor Ets1 suppresses T follicular helper type 2 cell differentiation to halt the onset of systemic lupus erythematosus. *Immunity*, 49(6): 1034–1048.e8.

https://doi.org/10.1016/j.immuni.2018.10.012

25. Sunshine A, Goich D, Stith A, *et al*., 2019, Ets1 controls the development of B cell autoimmune responses in a cell-intrinsic manner. *Immunohorizons*, 3(7): 331–340.

https://doi.org/10.4049/immunohorizons.1900033

26. Xia Y, Tao JH, Fang X, *et al*., 2018, MicroRNA-326 upregulates B cell activity and autoantibody production in lupus disease of MRL/lpr mice. *Mol Ther Nucleic Acids*, 11: 284–291.

https://doi.org/10.1016/j.omtn.2018.02.010

27. Chen L, Huang Z, Yang B, *et al*., 2015, Association of E26 transformation specific sequence 1 variants with rheumatoid arthritis in Chinese han population. *PLoS One*, 10(8): e0134875.

https://doi.org/10.1371/journal.pone.0134875

28. Yang B, Luo L, Chen L, *et al*., 2021, ETS1 polymorphism rs73013527 in relation to serum RANKL levels among patients with RA. *Medicine* (*Baltimore*), 100(5): e24562.

https://doi.org/10.1097/MD.0000000000024562

29. Yan M, Komatsu N, Muro R, *et al*., 2022, ETS1 is a key transcription factor that drives RANKL-expressing, tissue-destructive fibroblasts. *Nat Immunol*, 23(9): 1303–1304.

https://doi.org/10.1038/s41590-022-01298-9

30. Zhao N, Zou H, Qin J, *et al*., 2018, MicroRNA-326 contributes to autoimmune thyroiditis by targeting the Ets-1 protein. *Endocrine*, 59(1): 120–129.

https://doi.org/10.1007/s12020-017-1465-4

31. Zhao N, Wang Z, Cui X, *et al*., 2021, *In vivo* inhibition of MicroRNA-326 in a NOD.H-2(h4) mouse model of autoimmune thyroiditis. *Front Immunol*, 12: 620916.

https://doi.org/10.3389/fimmu.2021.620916

32. Li L, Ma X, Zhao YF, *et al*., 2020, MiR-1-3p facilitates Th17 differentiation associating with multiple sclerosis via targeting ETS1. *Eur Rev Med Pharmacol Sci*, 24(12): 6881–6892.

https://doi.org/10.26355/eurrev_202006_21678

33. Luo Y, Yang H, Wan Y, *et al*., 2022, Endothelial ETS1 inhibition exacerbate blood-brain barrier dysfunction in multiple sclerosis through inducing endothelial-to-mesenchymal transition. *Cell Death Dis*, 13(5): 462.

https://doi.org/10.1038/s41419-022-04888-5

34. Buggy Y, Maguire TM, McGreal G, *et al*., 2004, Overexpression of the Ets-1 transcription factor in human breast cancer. *Br J Cancer*, 91(7): 1308–1315.

https://doi.org/10.1038/sj.bjc.6602128

35. Li Y, Wu T, Peng Z, *et al*., 2022, ETS1 is a prognostic biomarker of triple-negative breast cancer and promotes the triple-negative breast cancer progression through the YAP signaling. *Am J Cancer Res*, 12(11): 5074–5084.

36. Fang LW, Kao YH, Chuang YT, *et al*., 2019, Ets-1 enhances tumor migration through regulation of CCR7 expression. *BMB Rep*, 52(9): 548–553.

https://doi.org/10.5483/BMBRep.2019.52.9.232

37. Peng P, Ren Y, Wan F, *et al*., 2023, Sculponeatin A promotes the ETS1-SYVN1 interaction to induce SLC7A11/xCT-dependent ferroptosis in breast cancer. *Phytomedicine*, 117: 154921.

https://doi.org/10.1016/j.phymed.2023.154921

38. Furlan A, Vercamer C, Heliot L, *et al*., 2019, Ets-1 drives breast cancer cell angiogenic potential and interactions between breast cancer and endothelial cells. *Int J Oncol*, 54(1): 29–40.

https://doi.org/10.3892/ijo.2018.4605

39. Ballschmieter P, Braig M, Lindemann RK, *et al*., 2003, Splicing variant DeltaVII-Ets1 is downregulated in invasive Ets1-expressing breast cancer cells. *Int J Oncol*, 22(4): 849–853.

https://doi.org/10.3892/ijo.22.4.849

40. Furlan A, Vercamer C, Bouali F, *et al*., 2014, Ets-1 controls breast cancer cell balance between invasion and growth. *Int J Cancer*, 135(10): 2317–2328.

https://doi.org/10.1002/ijc.28881

41. Kim GC, Lee CG, Verma R, *et al*., 2020, ETS1 suppresses tumorigenesis of human breast cancer via trans-activation of canonical tumor suppressor genes. *Front Oncol*, 10: 642.

https://doi.org/10.3389/fonc.2020.00642

42. Nazir SU, Kumar R, Singh A, *et al*., 2019, Breast cancer invasion and progression by MMP-9 through Ets-1

transcription factor. *Gene*, 711: 143952.

https://doi.org/10.1016/j.gene.2019.143952

43. Ito H, Duxbury M, Benoit E, *et al.*, 2004, Prostaglandin E2 enhances pancreatic cancer invasiveness through an Ets-1-dependent induction of matrix metalloproteinase-2. *Cancer Res*, 64(20): 7439–7446.

https://doi.org/10.1158/0008-5472.CAN-04-1177

44. Gao H, Peng C, Liang B, *et al.*, 2014, beta6 integrin induces the expression of metalloproteinase-3 and metalloproteinase-9 in colon cancer cells via ERK-ETS1 pathway. *Cancer Lett*, 354(2): 427–437.

https://doi.org/10.1016/j.canlet.2014.08.017

45. Tsai CL, Jung SM, Chi LM, *et al.*, 2021, Glycogen synthase kinase-3 beta (GSK3beta)-mediated phosphorylation of ETS1 promotes progression of ovarian carcinoma. *Aging* (*Albany NY*), 13(10): 13739–13763.

https://doi.org/10.18632/aging.202966

46. Phuchareon J, McCormick F, Eisele DW, *et al.*, 2015, EGFR inhibition evokes innate drug resistance in lung cancer cells by preventing Akt activity and thus inactivating Ets-1 function. *Proc Natl Acad Sci U S A*, 112(29): E3855–E3863.

https://doi.org/10.1073/pnas.1510733112

47. Seidel JJ, Graves BJ, 2002, An ERK2 docking site in the Pointed domain distinguishes a subset of ETS transcription factors. *Genes Dev*, 16(1): 127–137.

https://doi.org/10.1101/gad.950902

48. Myers E, Hill AD, Kelly G, *et al.*, 2005, Associations and interactions between Ets-1 and Ets-2 and coregulatory proteins, SRC-1, AIB1, and NCoR in breast cancer. *Clin Cancer Res*, 11(6): 2111–2122.

https://doi.org/10.1158/1078-0432.CCR-04-1192

49. Tomar S, Plotnik JP, Haley J, *et al.*, 2018, ETS1 induction by the microenvironment promotes ovarian cancer metastasis through focal adhesion kinase. *Cancer Lett*, 414: 190–204.

https://doi.org/10.1016/j.canlet.2017.11.012

50. Ning S, Chao HJ, Li S, *et al.*, 2022, The auto-inhibition mechanism of transcription factor Ets-1 induced by phosphorylation on the intrinsically disordered region. *Comput Struct Biotechnol J*, 20: 1132–1141.

https://doi.org/10.1016/j.csbj.2022.02.025

51. Wei W, Hu Z, Fu H, *et al.*, 2012, MicroRNA-1 and microRNA-499 downregulate the expression of the ets1 proto-oncogene in HepG2 cells. *Oncol Rep*, 28(2): 701–706.

https://doi.org/10.3892/or.2012.1850

52. Ma N, Chen F, Shen SL, *et al.*, 2015, MicroRNA-129-5p inhibits hepatocellular carcinoma cell metastasis and invasion via targeting ETS1. *Biochem Biophys Res Commun*, 461(4): 618–623.

https://doi.org/10.1016/j.bbrc.2015.04.075

53. Xu C, Liu S, Fu H, *et al.*, 2010, MicroRNA-193b regulates proliferation, migration and invasion in human hepatocellular carcinoma cells. *Eur J Cancer*, 46(15): 2828–2836.

https://doi.org/10.1016/j.ejca.2010.06.127

54. Allam MM, Diab KA, Khalil FO, *et al.*, 2022, The association between micro-RNA gene polymorphisms and the development of hepatocellular carcinoma in Egyptian patients. *Arch Med Sci*, 18(1): 62–70.

https://doi.org/10.5114/aoms/100600

55. Zheng L, Qi T, Yang D, *et al.*, 2013, microRNA-9 suppresses the proliferation, invasion and metastasis of gastric cancer cells through targeting cyclin D1 and Ets1. *PLoS One*, 8(1): e55719.

https://doi.org/10.1371/journal.pone.0055719

56. Zheng L, Pu J, Qi T, *et al.*, 2013, miRNA-145 targets v-ets erythroblastosis virus E26 oncogene homolog 1 to suppress the invasion, metastasis, and angiogenesis of gastric cancer cells. *Mol Cancer Res*, 11(2): 182–193.

https://doi.org/10.1158/1541-7786.MCR-12-0534

57. Zhang Y, Yan LX, Wu QN, *et al.*, 2011, miR-125b is methylated and functions as a tumor suppressor by regulating the ETS1 proto-oncogene in human invasive breast cancer. *Cancer Res*, 71(10): 3552–3562.

https://doi.org/10.1158/0008-5472.CAN-10-2435

58. Li W, Zang W, Liu P, *et al.*, 2014, MicroRNA-124 inhibits cellular proliferation and invasion by targeting Ets-1 in breast cancer. *Tumour Biol*, 35(11): 10897–10904.

https://doi.org/10.1007/s13277-014-2402-2

59. Wang R, Ma Y, Yu D, *et al.*, 2015, miR-377 functions as a tumor suppressor in human clear cell renal cell carcinoma by targeting ETS1. *Biomed Pharmacother*, 70: 64–71.

https://doi.org/10.1016/j.biopha.2015.01.012

60. Hua S, Lei L, Deng L, *et al.*, 2018, miR-139-5p inhibits aerobic glycolysis, cell proliferation, migration, and invasion in hepatocellular carcinoma via a reciprocal regulatory interaction with ETS1. *Oncogene*, 37(12): 1624–1636.

https://doi.org/10.1038/s41388-017-0057-3

61. Zhai W, Ma J, Zhu R, *et al.*, 2018, MiR-532-5p suppresses renal cancer cell proliferation by disrupting the ETS1-mediated positive feedback loop with the KRAS-NAP1L1/P-ERK axis. *Br J Cancer*, 119(5): 591–604.

https://doi.org/10.1038/s41416-018-0196-5

62. Jin L, Huang S, Guan C, *et al.*, 2020, ETS1-activated SNHG10 exerts oncogenic functions in glioma via targeting miR-532-3p/FBXL19 axis. *Cancer Cell Int*, 20(1): 589.

https://doi.org/10.1186/s12935-020-01649-2

63. Bukavina L, Bensalah K, Bray F, *et al*., 2022, Epidemiology of renal cell carcinoma: 2022 update. *Eur Urol*, 82(5): 529–542.

https://doi.org/10.1016/j.eururo.2022.08.019

64. Yang X, Zhang Y, Fan H, 2021, Downregulation of SBF2-AS1 functions as a tumor suppressor in clear cell renal cell carcinoma by inhibiting miR-338-3p-targeted ETS1. *Cancer Gene Ther*, 28(7–8): 813–827.

https://doi.org/10.1038/s41417-020-0197-4

65. Pan G, Zhang J, You F, *et al*., 2022, ETS proto-oncogene 1-activated muskelin 1 antisense RNA drives the malignant progression of hepatocellular carcinoma by targeting miR-22-3p to upregulate ETS Proto-Oncogene 1. *Bioengineered*, 13(1): 1346–1358.

https://doi.org/10.1080/21655979.2021.2017565

66. Sun Y, Zhang H, Ma R, *et al*., 2023, ETS-1-activated LINC01016 over-expression promotes tumor progression via suppression of RFFL-mediated DHX9 ubiquitination degradation in breast cancers. *Cell Death Dis*, 14(8): 507.

https://doi.org/10.1038/s41419-023-06016-3

67. Vallarelli AF, Rachakonda PS, Andre J, *et al*., 2016, TERT promoter mutations in melanoma render TERT expression dependent on MAPK pathway activation. *Oncotarget*, 7(33): 53127–53136.

https://doi.org/10.18632/oncotarget.10634

68. Li Y, Zhou QL, Sun W, *et al*., 2015, Non-canonical NF-kappaB signalling and ETS1/2 cooperatively drive C250T mutant TERT promoter activation. *Nat Cell Biol*, 17(10): 1327–1338.

https://doi.org/10.1038/ncb3240

69. Kohli JS, Mir H, Wasif A, *et al*., 2017, ETS1, nucleolar and non-nucleolar TERT expression in nevus to melanoma progression. *Oncotarget*, 8(61): 104408–104417.

https://doi.org/10.18632/oncotarget.22254

70. Xu X, Li Y, Bharath SR, *et al*., 2018, Structural basis for reactivating the mutant TERT promoter by cooperative binding of p52 and ETS1. *Nat Commun*, 9(1): 3183.

https://doi.org/10.1038/s41467-018-05644-0

71. Gabler L, Lotsch D, Kirchhofer D, *et al*., 2019, TERT expression is susceptible to BRAF and ETS-factor inhibition in BRAF(V600E)/TERT promoter double-mutated glioma. *Acta Neuropathol Commun*, 7(1): 128.

https://doi.org/10.1186/s40478-019-0775-6

72. Dittmer J, 2003, The biology of the Ets1 proto-oncogene. *Mol Cancer*, 2: 29.

https://doi.org/10.1186/1476-4598-2-29

73. Priebe V, Sartori G, Napoli S, *et al*., 2020, Role of ETS1 in the transcriptional network of diffuse large B cell lymphoma of the activated B cell-like type. *Cancers* (*Basel*), 12(7): 1912.

https://doi.org/10.3390/cancers12071912

74. Overbeck BM, Martin-Subero JI, Ammerpohl O, *et al*., 2012, ETS1 encoding a transcription factor involved in B-cell differentiation is recurrently deleted and down-regulated in classical Hodgkin's lymphoma. *Haematologica*, 97(10): 1612–1614.

https://doi.org/10.3324/haematol.2012.061770

75. Bonetti P, Testoni M, Scandurra M, *et al*., 2013, Deregulation of ETS1 and FLI1 contributes to the pathogenesis of diffuse large B-cell lymphoma. *Blood*, 122(13): 2233–2241.

https://doi.org/10.1182/blood-2013-01-475772

76. Chung EYL, Sartori G, Ponzoni M, *et al*., 2023, ETS1 phosphorylation at threonine 38 is associated with the cell of origin of diffuse large B cell lymphoma and sustains the growth of tumour cells. *Br J Haematol*, 203(2): 244–254.

https://doi.org/10.1111/bjh.19018

77. McCarter AC, Della Gatta G, Melnick A, *et al*., 2020, Combinatorial ETS1-dependent control of oncogenic NOTCH1 enhancers in T-cell leukemia. *Blood Cancer Discov*, 1(2): 178–197.

https://doi.org/10.1158/2643-3230.BCD-20-0026

78. Luchtel RA, Zhao Y, Aggarwal RK, *et al*., 2022, ETS1 is a novel transcriptional regulator of adult T-cell leukemia/lymphoma of North American descent. *Blood Adv*, 6(20): 5613–5624.

https://doi.org/10.1182/bloodadvances.2022007725

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

REVIEW ARTICLE

Cyanine-induced apoptosis for cancer therapy

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Abstract

Apoptosis is a programmed cell death characterized by cell rounding, chromatin condensation and clumping, cytoplasmic collapse, and the formation of apoptotic bodies. Recently, increasing scientific research has disclosed the close relationship between apoptosis and cyanine. Cyanine, due to its excellent fluorescent characteristics, is used in constructing vital fluorescent cores for fluorescent probes. It has been extensively utilized in targeted labeling for growing cells and tumor phototherapy. Cyanine-derived compounds have provided new approaches for treating different cancers by inducing tumor cell apoptosis. This review introduces the molecular mechanism of apoptosis, essential apoptosis-related proteins, and the pathways related to tumor cell apoptosis. Furthermore, it systematically discusses the function of cyanine in cancer therapy by inducing apoptosis. A deeper understanding of cyanine-induced apoptosis may offer new options for cancer treatment and new strategies for developing and utilizing cyanine.

Keywords: Cancer; Apoptosis; Cyanine; Cytochrome C; Caspase3

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1. Introduction

As the human social economy grows and the standard of living improves, malignant tumors have become one of the three primary illnesses endangering human daily life and well-being[1,2]. They are a major cause of global death[3], with morbidity and mortality rates increasing in recent years. Traditional cancer therapies include exercise, radiotherapy, chemotherapeutics, combined radiation and chemotherapy, and immunization therapy. However, their therapeutic effect, targets, and degree of radical cure remain limited. Consequently, there is a pressing need to develop more sophisticated treatment modalities and anticancer drugs.

Achieving tumor cell death is the ultimate goal of anti-tumor remedy, and the induction of tumor cell apoptosis is an effective strategy for combating cancer[4].Cell death plays a significant role in both growth and aging processes, as deviations in cell mortality rates, whether excessively high or low, can precipitate a range of diseases[5-7]. Apoptosis, necrosis, autophagy, ferroptosis, cuproptosis, and pyroptosis are the

predominant modes of cell death (Table 1)[6,8]. Disorders in apoptotic mechanisms are associated with cancer growth and confer resistance against tumor therapy[9].

Apoptosis, recognized as the first identified type of cell death, is also known as type I programmed cell death[22,23]. Unlike other forms of cell death, which are not self-inflicted but rather self-protective and controlled by specific genes, apoptosis is a natural and regulated process essential for cellular development and death[6,24,25]. Apoptosis plays a significant role in the elimination of damaged or redundant cells[26], facilitated by a number of proteins through the enzymatic activity of effector cystathionases[27]. Apoptosis signal dysregulation and aberrant apoptosis are implicated in the pathogenesis of various diseases, including ectopic calcification[28], neurodegenerative disorders, autoimmune diseases, acquired immunodeficiency syndrome (AIDS), and local ischemia. Thus, apoptosis manifests as a double-edged sword phenomenon for cells[29]. Morphological changes in cells are the main characteristics defining apoptosis and include cell shrinkage[30], cell membrane blebbing, nuclear fragmentation, phosphatidylserine ectopia, nuclear sequestration, mRNA degradation, and apoptotic vesicle formation[6,31-34]. From another perspective, apoptosis is considered an indispensable biomarker for certain malignancies. Upregulation of pro-apoptotic gene expression and downregulation of antiapoptotic gene expression can better predict the survival time of cancer patients[35,36].

As bilayer membrane organelles, mitochondria play a pivotal role in regulating cell apoptosis and serve as the primary sites for the generation of reactive oxygen species (ROS)[37,38]. Mitochondria undergo dramatic morphological and biochemical changes during apoptosis, including mitochondrial fission and fusion kinetics[22,23]. This process involves both division and fusion, with an increase in fission activity and a decrease in fusion activity, thus inducing apoptosis. However, mitochondrial changes during apoptosis closely correlate with cancer malignancy and treatment effectiveness[39]. Mitochondria acts as centers for various stressors, and the combination of different advent stress signals often precedes the manifestation of apoptotic morphology[30]. ROS can induce intracellular changes in mitochondrial outer membrane permeability (MOMP) by acting on mitochondrial membranes[6], which are monitored by proteins in the B cell lymphoma 2 (Bcl-2) family, including pro-apoptotic and antiapoptotic proteins, whose balance determines cell death and survival[38]. Cytochrome C (Cyto C) is released from mitochondria, activating the cysteine enzyme cascade reaction, thereby inducing apoptosis[23]. Mitochondria amplify the activation of cysteine proteases during apoptosis by releasing Cyto c and other cofactors, accompanied by organelle breakage and cristae remodeling[40]. Dysfunctional mitochondria have implications for many diseases[41], such as malignant tumors, obesity, glycuresis, and neurodegenerative diseases. Several studies have demonstrated that the development

Table 1. Classification of cell death patterns

Types of cell death	Key proteins	Signaling pathways	Morphological features	Biochemical features	References
Apoptosis	Caspase, Bcl-2, BAX, P53, FAS, Cytochrome C, BAX	PTEN/PI3K/AKT, AMPK/AKT, MAPK/JNK, AKT/BAD/Bcl-2	Chromatin condensation, karyorrhexis and formation of apoptotic bodies	DNA fragmentation	[10,11]
Necroptosis	RIP1, RIP3, MLKL, P38, PGAM5	RIP1/RIP3/MLKL, PKC-MAPK-AP-1	Plasma membrane rupture, cytoplasmic swelling	ATP level decrease	[12,13]
Autophagy	mTOR, LC3II, Beclin-1, DRAM3, TFEB, P62, ULK1	mTOR, Beclin-1, P53, MAPK	Formation of double membrane self-solution	Lysosome activity increased	[14]
Ferroptosis	GPX4, TFR1, SLC7A11, NRF2, NCOA4, P53, Ferritin	Xc-/GPX4, P53/SLC7A11	Mitochondria decrease or disappear, and the outer membrane ruptures	Iron accumulation	[15-17]
Pyroptosis	Caspase, NLRP3, GSDMD, IL-1β, IL-18, IL-1α, IL-6, IL-8	NF-κB	Infiltration of extracellular contents and cell swelling	The formation of inflammatory vesicles	[18-21]

Abbreviations: AKT: Protein kinase B; AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase; ATP: Adenosine triphosphate; BAD: Bcl-xl/Bcl-2-associated death promoter; BAX: Bcl-2-associated X protein; Bcl-2: B cell lymphoma 2; DRAM-1: Damage-regulated autophagy modulator; FAS: Apoptosis stimulating fragment; GPX4: Glutathione peroxidase 4; GSDMD: Gasdermin D; IL: Interleukin;
JNK: Recombinant c-Jun N-terminal kinase; LC3II: Light chain 3-II; MAPK: Mitogen-activated protein kinase; MLKL: Mixed lineage kinase domain-like; mTOR: Mammalian target of rapamycin; NCOA4: Nuclear receptor coactivator 4; NF-κB: Nuclear factor kappa B; NLRP3: NOD-like receptor (NLR) family pyrin domain-containing 3; Nrf2: Nuclear factor E2-related factor 2; P53: Protein 53; P62: Protein 62; PGAM5: Phosphoglycerate mutase 5; PI3K: Phosphatidylinositol 3-kinase; PTEN: Phosphatase and tensin homolog deleted on chromosome ten; RIP: Receptor-interacting protein; SLC7A11: Cystine transporter solute carrier family 7 member 11; TFEB: Transcriptional factor EB; TfR1: Transferrin receptor 1; ULK1: Unc-51 like kinase 1.

of cancer at different stages involves alterations in mitochondrial function[42-45], making mitochondria one of the key targeted organelles for anti-cancer therapy (Figure 1)[46].

In recent years, cyanine has been widely employed in anti-tumor research. These compounds are classified based on their chemical composition into Cy3, Cy5, and Cy7, depending on the number of carbon atoms they contain. For example, Cy7, a type of cyanine compound, has demonstrated the ability to produce ROS under a specific wavelength of radiation. This property opens up the possibility of using Cy7 to induce apoptosis in tumor cells. Cy7, as a novel fluorescent dye with anti-cancer properties, holds the potential to reduce the dosage of chemotherapy drugs, mitigate their toxic side effects, and treat tumor cells through the combination of chemotherapy, near-infrared fluorescence imaging (NIRF), and photothermal therapy (PTT)[47]. Therefore, a thorough investigation into the relationship between cyanine and apoptosis is imperative.

2. Mechanism of apoptosis

Apoptosis can be initiated through two different pathways: the Bcl-2-regulated mitochondrial cystatin-dependent pathway and the exogenous apoptotic signaling pathway mediated by cell membrane proteins of the death receptor[22,48,49].

2.1. Intrinsic apoptosis

Mitochondrial-mediated apoptosis is the significant pathway for cell death[50]. Intrinsic pathways can be induced by various intracellular stress stimuli, eventually leading to MOMP, release of Cyto C, formation and activation of caspase-9, and increased ROS levels[8,26]. Cells sense intracellular stressors such as growth factors, nutrient deficiencies, DNA damage, UV radiation, and hypoxia, and induce apoptosis by initiating MOMP[6]. During MOMP, Cyto C is released from mitochondria[51], promoting the oligomerization of apoptotic protease activating factor 1 (Apaf-1), and forming the heptamer. Apaf-1 contains a, which combines with pro-caspase-9 to form the apoptosome, a large complex of Cyto C, Apaf-1, and pro-caspase-9[52-56]. This pathway relies on the release of Cyto C from mitochondria, requiring a high ratio of BAX (Bcl-2 associated X protein)/Bcl-2 (B cell lymphoma 2) to create favorable conditions for BAX insertion into mitochondria and subsequent Cyto C release[10]. Cyto C then recruits caspase-9 to form the apoptosome complex. Ultimately, activation of caspase-3 initiates the caspase cascade[26].

Mitochondrial outer membrane permeabilization is significant in intrinsic apoptosis, which is regulated by the

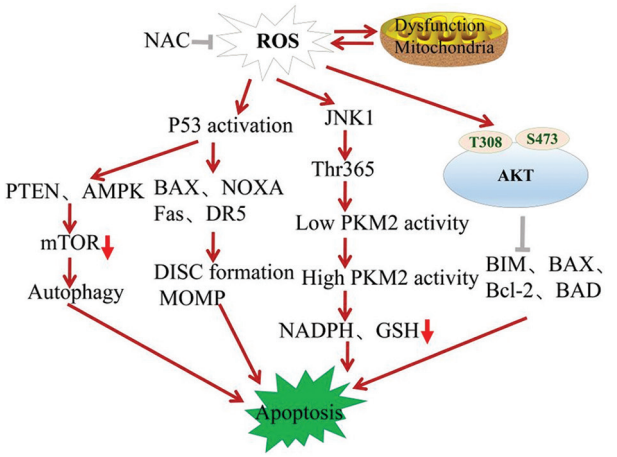

Figure 1. The role of mitochondria in apoptosis. Mechanistically, mitochondria serve as the primary sites for producing ROS during apoptosis. These ROS can induce changes in intracellular MOMP by acting on the mitochondrial membrane, which is regulated by proteins in the Bcl-2 family. Subsequently, the release of Cyto C from mitochondria activates cysteine cascades, leading to cell apoptosis.
Abbreviations: AKT: Protein kinase B; AMPK: Adenosine 5'-monophosphate(AMP)-activated protein kinase; BAD: Bcl-xl/Bcl-2 associated death promoter; BAX: Bcl-2 associated X protein; Bcl-2: B cell lymphoma 2; BIM: Bcl-2 interacting mediator of cell death; DISC: Death-inducing signaling complex; DR5: Death receptor 5; FAS: Apoptosis stimulating fragment; GSH: glutathione (L-gamma-Glutamyl-L-cysteinyl-glycine); JNK1: Recombinant c-Jun N-terminal kinase 1; MOMP: Mitochondrial outer membrane permeabilization; mTOR: Mammalian target of rapamycin; NAC: N-Acetyl-L-cysteine; NADPH: Nicotinamide adenine dinucleotide phosphate; P53: Protein 53; PKM2: Active Pyruvate kinase isozymes M2; PTEN: Phosphatase and tensin homolog deleted on chromosome ten; ROS: Reactive oxygen species.

interaction of multiple proteins from the members of the pro-apoptotic Bcl-2 family, such as Bcl-2, BAX, and the Bcl-2 antagonist/killer 1 (BAK)[57]. MOMP is essential for inducing the release of apoptosis-inducing factor (AIF)[39], which subsequently translocates freely into the cytoplasm or nucleus, causing DNA breakage and ultimately mediating apoptosis. The intrinsic pathway begins with the release of Cyto C into the cytoplasm, followed by its activation by pro-apoptotic proteins[58]. The release of free Cyto C into the cytoplasm is a crucial requirement for triggering apoptosis and is regarded as a point of no return in cell death, occurring independently of cystathionin activation (Figure 2)[54,59,60].

2.2. Extrinsic apoptosis

Extrinsic apoptosis, mediated by cell membrane proteins of the death receptor[27], is the second major pathway of apoptosis, initiated by extracellular signals originating from the immune system or the external environment[8]. The tumor necrosis factor (TNF) family receptors, consisting of apoptosis stimulating fragment (FAS)-R,

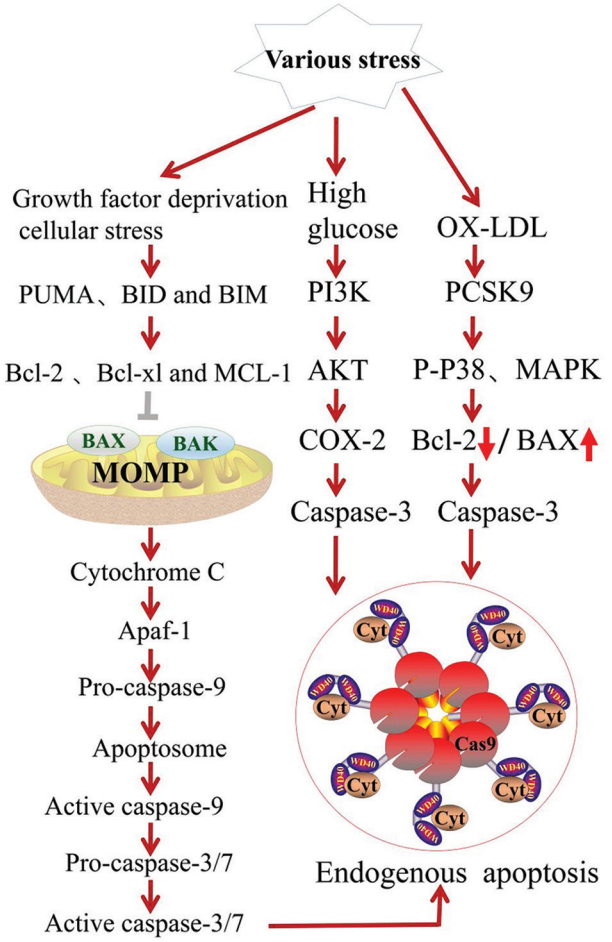

Figure 2. The mechanism of endogenous apoptosis in cells. Various intracellular stress stimuli can induce apoptosis, ultimately leading to MOMP, release of Cyto C, formation and activation of caspase-9, and an increase in ROS. During MOMP, free mitochondrial Cyto C translocates into the cytoplasm, resulting in the oligomerization of Apaf-1 and the formation of a heptamer. Caspase-9 is subsequently captured by Cyto C, leading to the formation of apoptosome and ultimately resulting in the activation of caspase-3, which triggers other caspase pathways immediately. The intrinsic pathway begins with the release of Cyto C into the cytoplasm, followed by its activation by pro-apoptotic proteins, ultimately leading to apoptosis.
Abbreviations: AKT: Protein kinase B; BAK: Bcl-2 antagonist/killer 1; BAX: Bcl-2 associated X protein; Bcl-2: B cell lymphoma 2; Bcl-xl: B-cell leukemia/lymphoma xl; BID: Bcl-2 homology 3 interacting domain death agonist; Cas9: Caspase-9; COX-2: Cyclooxygenase-2; Cyt: Cytochrome; Cyto C: Cytochrome C; MAPK: Mitogen-activated protein kinase; MCL-1: Myeloid cell leukemia-1; OX-LDL: Oxidized low-density lipoprotein; P-P38: Phosphorylation-p38; PCSK9: Proprotein convertase subtilisin/kexin type 9; PI3K: Phosphatidylinositol 3-kinase; PUMA: p53 upregulated modulator of apoptosis.

TRAIL-R1, TRAIL-R2, TNF-R1, and TNF-R2, along with their respective ligands, bind to the FAS-associated protein with death domain (FADD), caspase-8 and caspase-10 to form death-inducing signaling complex (DISC)[27], which leads to caspase-3 cleavage and apoptosis. Formation of DISC directly increases MOMP, resulting in the release of Cyto C into the cytosol. Cyto C then combines with Apaf-1, leading to the formation of apoptosome, which activates caspase-9. Activation of caspase-9 further activates caspase-3, ultimately inducing cell apoptosis (Figure 3)[61-63].

2.3. Commonalities of intrinsic and extrinsic apoptosis

Both intrinsic and extrinsic apoptosis share common effectors, including caspase-3 and caspase-7, which are essential to apoptosis[64,65]. These effectors participate in and mediate multiple steps of apoptosis, ultimately leading to the exposure of phosphatidylserine ("eat me" signal)[8,66-68]. Furthermore, caspase-3 and caspase-7 activate several other precursors to cystathionine (such as caspase-2, caspase-6, caspase-8, and caspase-10) by hydrolyzing their proteins into active forms, thus amplifying apoptotic signals further[69-73].

3. The main apoptosis-related proteins

3.1. Classification of apoptosis proteins

Apoptosis is a complex process involving intricate cellular proteins and signal transduction cascades. Two types of apoptotic proteins exist, classified based on their role: pro-apoptotic and antiapoptotic. The equilibrium between these apoptotic proteins is crucial in determining whether apoptosis occurs in cells[9]. Caspases, members of the cysteine protease family, are classified into three types based on their function and structure: (i) inflammatory caspase, including caspase-1, -4, -5, -11, -12, -13, and -14; (ii) initiating caspase, including caspase-2, caspase-8, caspase-9, and caspase-10; and (iii) effector caspase, including caspase-3, caspase-6, and caspase-7[74,75]. The Bcl-2 family, which exhibits both pro-apoptotic and antiapoptotic effects and is located outside mitochondria[76,77], plays a crucial role in maintaining the balance between pro-apoptotic and antiapoptotic proteins, governing the sensitivity of cells to apoptotic stimuli[78]. The Bcl-2 family is categorized into three types: (i) pro-apoptotic proteins (BAX, BAK, and BOK), which are involved in targeting and permeabilizing outer mitochondrial membranes, promoting the release of free Cyto C into the cytoplasm to maintain mitochondrial integrity and inhibit damage to mitochondrial membrane potential[79]; (ii) antiapoptotic Bcl-2 family, including Bcl-2 and Bcl-xl, which inhibits MOMP and can block Cyto c release[6]; and (iii) Bcl-2 homologous 3 proteins (BH3), such as p53 upregulated modulator of apoptosis (PUMA), Bcl-2 homology 3 interacting domain death agonist (BID), and Bcl-2 interacting mediator of cell death (BIM), which promote apoptosis[80-82]. Primarily, the Bcl-2 protein family regulates intrinsic (mitochondrial) pathways (Table 2)[83,84].

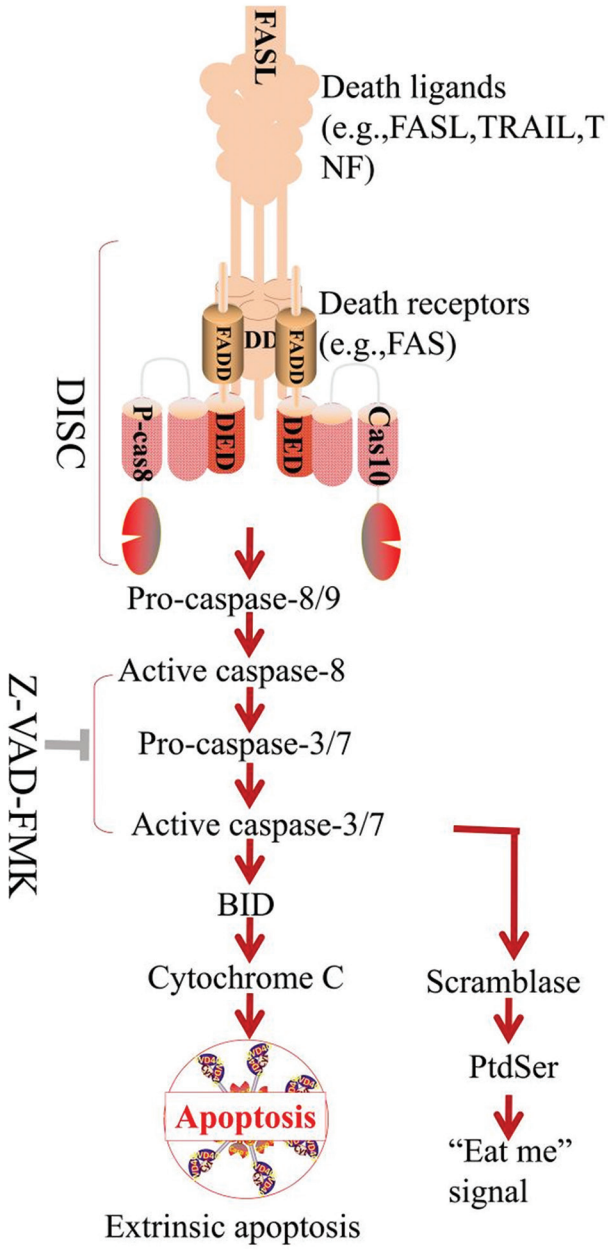

Figure 3. The mechanism of extrinsic cell apoptosis. The mechanism of extrinsic cell apoptosis involves the mediation of cell membrane proteins of the death receptors. This process is initiated when members of the TNF family receptors and their ligands, composed of FAS (FAS-L), TRAIL (TRAIL-R1 and TRAIL-R2), and TNF (TNF-R1 and TNF-R2), bind to FADD, caspase-8, and caspase-10 proteins, respectively, to form DISC, which directly increases MOMP. After the release of free Cyto C into the cytoplasm, Cyto C binds with Apaf-1, leading to the formation of an apoptosome, activating caspase-9 and eventually leading to caspase-3 cleavage and apoptosis.

Abbreviations: BID: BH3 interacting domain death agonist; Cas10: Caspase-10; Cyto C: Cytochrome C; DED: Death effector domain; DISC: Death-inducing signaling complex; FADD: FAS-associated protein with death domain; FASL: Recombinant factor-related apoptosis ligand; MOMP: Mitochondrial outer membrane permeability; P-cas8: Pro-caspase-8; PtdSer: Phosphatidylserine; TNF: Tumor necrosis factor; TRAIL: TNF-related apoptosis-inducing ligand.

3.2. Pro-apoptotic proteins

3.2.1. BAX and BAK

BAX and BAK, pro-apoptotic members of the Bcl-2 family, cause MOMP by forming large pores in the outer mitochondrial membrane through conformational changes and oligomerization[91,92]. Dysregulation of BAX, often through suppression or mutation, is commonly observed in cancer, with reduced expression of BAX associated with poorer prognosis[93,94]. In addition, deficiency in BAK, along with BAX, significantly inhibits mitochondrial-mediated apoptotic cell death[95]. Studies have identified pro-apoptotic BAX mutations in colorectal cancer (CRC), which contribute to resistance against anti-cancer therapies[96]. Alterations in the BCL-2/BAX ratio have also been implicated in chronic lymphocytic leukemia[97]. Furthermore, intrinsic pathways can be disrupted in cancer through mechanisms such as reduced expression of Apaf-1in melanoma due to abnormalities in promoter methylation[98,99].

3.2.2. Cytochrome C

Cyto C, located within the mitochondrial, is a small spherical core-coding protein containing covalently linked heme groups. Studies on Cyto C-deficient BALB/C nude mice have revealed that these mice die during mid-gestation, suggesting that Cyto C is a protein essential for organism development and mitochondrial adenosine triphosphate (ATP) production[100]. Functioning as a crucial signaling molecule, Cyto C initiates apoptosis by activating downstream cysteine cascades. The BAX/BAK pore mediates the release of Cyto C and mitochondrial DNA (mtDNA) from the mitochondria[39]. Moreover, Cyto C plays an irreplaceable role in ATP generation and cell survival. Through its interaction with Apaf-1, Cyto-C forms an active apoptosome, thereby initiating caspase-9 activity and the downstream caspase cascade. Furthermore, Cyto C plays a vital role in both the clearance and generation of ROS[101-103]. In summary, Cyto C is an integral component in apoptosis.

3.2.3. Caspase family

Caspases are key factors associated with apoptosis, serving as both initiators and executors of cell death. Major initiator caspases include caspase-2, -8, -9, and -10, while executors caspases include caspase-3, caspase-6, and caspase-7, which are accountable for cleaving cellular components[104]. Various regulators of apoptosis modulate the caspase enzyme system. Notably, caspase-3 (CPP32, YAMA, or apopain) stands out as the most relevant enzyme in apoptosis[105,106]. Caspase-3 plays a pivotal role in cleaving numerous critical proteins, such as poly(ADP-ribose) polymerase (PARP), leading to the activation of cysteine aspartate proteases through the cleavage of critical cellular substrates, which, in turn, results in important morphological changes in apoptosis[107]. Cleaved

Table 2. Classification of apoptotic proteins

Classification of apoptotic proteins	Specific families	Specific proteins	Function	References
Pro-apoptotic proteins	Caspase family	Caspase-2, -8, -10	Apoptosis activator caspases	[74]
		Caspase-3, -6, -7	Downstream apoptosis executioner caspase	[74]
		Caspase-1, -4, -5	Downstream substrates of inflammatory mediator caspases	[74]
	Apaf-1		A critical molecule in the intrinsic or the mitochondrial signal way of apoptosis	[85]
	Bcl-2 family	Bcl-xs, BAK 2, BAK 3, BAD, BID, BIK, HRK		[86]
	FAS		Caspase activator	[87]
	P53		Monitor and induce apoptosis of abnormal cells	[88]
	Myc		Promote cell proliferation or apoptosis	[89]
	ATM		DNA damage test	[90]
Anti-apoptotic proteins	Bcl-2 family		Bcl-2, Bcl-xl, Bcl-w, MCL-1, A 1, Bfl-1	[86]

Abbreviations: Apaf-1: Apoptotic protease activating factor 1; ATM: Ataxia telangiectasia mutated kinase; BAD: Bcl-xl/Bcl-2-associated death promoter; BAK: Bcl-2 antagonist/killer 1; Bcl: B cell lymphoma; BID: Bcl-2 homology 3 interacting domain death agonist; BIK: BCL-2 interacting killer; FAS: Apoptosis stimulating fragment; MCL-1: Myeloid cell leukemia-1.

caspase-3 is the predominant lytic enzyme that promotes apoptosis[108,109]. Previous studies have detected caspase-3 in a considerable number of human cancers, including non-small cell lung cancer[110], esophageal squamous carcinoma, and gastric cancer[111,112]. Recent studies have demonstrated that RNA interference (RNAi) technology can rapidly and specifically silence caspase-3 gene expression, offering a new option for antiapoptotic therapy[113].

3.3. Antiapoptotic proteins

3.3.1. BCL-2

Bcl-2, functioning as a proto-oncogene, serves as a pivotal regulator for cell death, inhibiting apoptosis and being closely related to the pathogenesis and resistance to various anticancer drugs[114-117]. Previous research has demonstrated that elevated levels of Bcl-2 in malignant cells can confer resistance to apoptosis induced by chemotherapy drugs such as cisplatin and arsenic trioxide[118-120]. Conversely, inhibition of Bcl-2 expression can trigger apoptosis in tumor cells. Overexpression of Bcl-2 has been associated with apoptosis resistance in a considerable number of human malignant tumors, including B-cell lymphoma, prostate carcinoma, and melanoma[49,121-124]. In addition, previous studies have revealed overexpression of Bcl-xl in CRC and Kaposi's sarcoma[125,126]. Therefore, the overexpression of Bcl-2 and Bcl-xl has been implicated in cisplatin resistance and malignant tumor recurrence rates across various cancers, including non-small cell lung, head-and-neck cancer, and breast cancers[127-131]. On the initiation of apoptosis, the scaffolding protein ARTS facilitates the formation of a ternary complex involving Bcl-2, ARTS, and XIAP. This complex allows XIAP to ubiquitinate Bcl-2 and degrade it by the proteasome[132], thereby highlighting the significant role of Bcl-2 in apoptosis.

3.3.2. Protein 53

The oncogene protein 53 (P53) plays a significant role in cancer by orchestrating diverse cellular processes such as cell cycle arrest, apoptosis, DNA repair, senescence metabolism, and antioxidant response[121,133,134]. Notably, the activation of P53 primarily leads to cell cycle arrest and apoptosis[121]. P53 is among the most frequently mutated or silenced genes in malignant tumors and has been extensively investigated in recent years. Its activation serves as a protective mechanism against tumorigenesis and promotes the efficacy of tumor eradication therapies. In response to cellular stress, P53 modulates both intrinsic and extrinsic apoptosis pathways[135]. Furthermore, P53 can directly influence mitochondria, participating in and inducing apoptosis[136]. Mechanistically, interactions between P53 and members of the Bcl-xl and pro-apoptotic Bcl-2 family liberate the pro-apoptotic effector BAX/BAK, thereby triggering the release of Cyto C and activating pro-caspase-3[137]. P53-induced apoptosis involves the regulation of apoptosis-associated proteins whose expression is controlled by P53. Furthermore, P53 induces apoptosis by initiating the release of Cyto C from mitochondria through mitochondrial translocation[138-140]. Mutations in the *p53* gene are present in more than half of all human malignancies. Introducing functional P53 into tumor cells containing mutant P53 can induce apoptosis and suppress tumor growth. Consequently, P53 is considered the most critical mediator of tumor cell apoptosis and is a key target in various anti-cancer therapies, including chemotherapy and radiation therapy[141,142].

P53 promotes apoptosis by upregulating the transcription of pro-apoptotic proteins, including death receptor 5 (DR5), FAS, and TNF-receptor type I (TNF-R1), leading to the activation of caspase-8[143,144]. Moreover, P53 induces the activation of PUMA, BAX, BID, Bcl-xl/Bcl-2-associated death promoter (BAD), BAK, and NOXA (PMAIP1)[82,145-147]. One mechanism by which P53 enhances cysteine protease activation is to induce BID, BAK, and BAX to increase MOMP[49,148]. The presence of P53 is significantly correlated with increased transcriptional activation of ARTS, a pro-apoptotic XIAP antagonist that counteracts XIAP inhibition of cysteine proteases, resulting in BID activation and mitochondrial outer membrane permeabilization[121,122]. Moreover, P53 plays a key role in germ cell apoptosis across various animals, including mammals and reptiles. Its mechanism involves the regulation of apoptosis-related proteins such as BID, BAX, Bcl-xl, and Bcl-2, as well as death receptors such as CD95, FAS, Apo-1, and DR5[149].

4. Signaling pathways of apoptosis

4.1. PTEN/PI3K/AKT

PTEN/PI3K/AKT plays a key role in apoptosis and is often activated in cancer[150,151]. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a dual protein that primarily dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate, and its activity is lost in human cancers[152]. PI3K, a lipid kinase, regulates various cellular processes and is implicated in chemotherapy resistance in cancer therapy[75]. AKT, a threonine/serine protein kinase, serves as a vital downstream effector in the PI3K/AKT signaling pathway. It exists in three forms: AKT1 (predominantly expressed in most tissues), AKT2 (mainly found in insulin-sensitive tissues such as the liver, pancreas, and muscle), and AKT3 (expressed in the cerebrum and orchis). Phosphorylation of Thr308 and Ser473 activates AKT, which in turn mediates apoptosis and cell cycle[153-158]. Numerous studies have illustrated a significant correlation between increased expression of Bcl-2 and aberrant activation of the PTEN/PI3K/AKT signaling pathway[159-162]. However, further investigation of this signaling pathway is essential for gaining insights and developing strategies for cancer therapy.

4.2. AMPK/AKT

Adenosine 5'-monophosphate(AMP)-activated protein kinase (AMPK) is an energetic cell sensor and plays an essential role in the apoptosis signaling pathway[163]. It exists as a heterotrimer complex comprised of α, β, and γ subunits, where β and γ serve as regulatory subunits while α serves as a catalytic subunit. Phosphorylation of threonine residue 172 on the α subunit is a significant process in activating AMPK[164,165]. AMPK is involved in various biological regulatory processes, including apoptosis, proliferation, and autophagy[166]. It regulates glucose and lipid metabolism through its interaction with ATP[167], adenosine diphosphate (ADP), and adenosine monophosphate (AMP), thereby modulating the mitochondrial energy of cells[168,169]. The disruption of oxidative phosphorylation results in a decrease in mitochondrial energy content, leading to an increase in the ADP/ATP or AMP/ATP ratio and subsequent activation of AMPK[154]. Surprisingly, AMPK may exert either pro- or anti-tumor effects depending on the metabolic environment[170]. Under conditions of metabolic stress, AMPK may promote tumorigenesis by stimulating alternative metabolic pathways such as mitochondrial phagocytosis and fatty acid combustion, thereby promoting metabolic plasticity. In addition, AMPK interacts with AKT, a key regulator of nutrient availability. Activation by AKT promotes glycolysis by upregulating hexokinase-2, which in turn affects mitochondrial permeability and apoptosis[171]. Furthermore, AKT can activate mTORC1/2, subsequently modulating downstream apoptosis signaling pathways[172-174].

4.3. MAPK/JNK

The mitogen-activated protein kinase (MAPK) pathway transmits extracellular signals to regulate apoptosis. Recent studies have presented different opinions on the role of the MAPK/JNK pathway in controlling apoptosis under genotoxic stress. Further understanding of the effect of MAPK/JNK on apoptosis regulation holds promise for early cancer treatment prognosis[175]. Phosphorylated (p)-JNK localizes to mitochondria, resulting in mitochondrial dysfunction characterized by decreased energy supply, disrupted MOMP, increased ROS production, and ultimately leading to apoptosis. This process involves the translocation of BAX to the outer mitochondrial membrane, leading to the widening of the pores in the outside membrane and apoptosis induction[162]. JNK, a member of the MAPK family, also known as stress-activated kinases, comprises three coding genes: JNK1, JNK2, and JNK3[176,177]. While JNK1 and JNK2 are generally expressed in various tissues, JNK3 is mainly expressed in the brain and heart[177,178]. Specific stimuli, such as MKK4 and MKK7, activate JNK through phosphorylation[179] and regulate the phosphorylation and activity of downstream factors[180-182]. In summary, MAPK/JNK plays a significant role in mitochondria-induced apoptosis[183].

5. Apoptosis induction based on cyanine

Cyanine was first discovered by Williams in 1856. It possesses fluorescence emission characteristics and serves as a fluorescent dye[184]. The chemical structure of cyanine consists of two components: a conjugated chain of multiple methylene groups and a cyclic structure, such as an aromatic, heterocyclic, or cyclic alkene, attached at the

ends or in the middle of the conjugated chain. Cyanines exhibit excellent properties, such as fluorescence features, including high molar absorbance, narrow absorption and emission bands, and compatibility with UV/VIS and near-infrared (NIR) regions[185-187]. Common cyanine dyes used for biomolecule labeling, such as DNA and proteins, include Cy3 (Figure 4A), Cy5 (Figure 4B), and Cy7 (Figure 4C). These dyes can induce apoptosis in cancer cells by producing ROS or high temperatures, thereby exerting an anti-tumor effect. Cyanine structures find widespread applications and can contribute to the anti-tumor effect by inducing apoptosis in cancer cells.

Photosensitive therapy presents a promising approach for tumor treatment, including photodynamic therapy (PDT) and PTT[188]. PDT, in particular, is a highly effective cancer treatment strategy due to its minimally invasive nature, effective targeted destruction of tumor cells, and the significant induction of systemic anti-tumor immunity[189]. This innovative method utilizes photosensitive drugs in combination with laser activation to target tumors. Photosensitizing drugs deliver energy to ambient oxygen, leading to the production of ROS, which exert toxicity and induce cell death[190]. Compared to traditional therapy, PDT offers advantages such as enhanced targeting, effective treatment outcomes, and reduced side effects[191,192]. Notably, PDT-induced apoptosis has been reported to release signaling molecules that trigger immune cell death, which is crucial for combating metastatic tumors and preventing recurrence[193].

5.1. Small molecule cyanine derivatives

Small molecule cyanine derivatives play an essential role in tumor therapy. They can exert an anti-tumor effect by accumulating within mitochondria, leading to the depolarization of the mitochondrial membrane. This accumulation triggers the production of ROS, which subsequently induces apoptosis or cell cycle arrest in cancer cells.

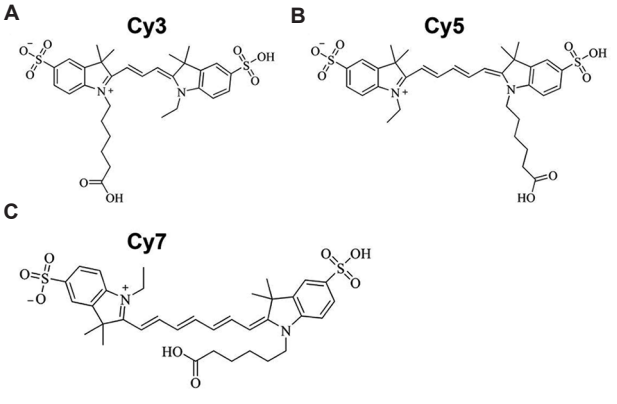

Figure 4. (A-C) Cyanine derivatives with different chain lengths. They are essential in tumor treatment and labeling.

5.1.1. Chemotherapy activity

Chemotherapy stands out as a significant approach in cancer treatment, and small molecule cyanine derivatives can contribute to this therapeutic strategy. Mitochondria, being pivotal subcellular organelles, serve as crucial targets for drug delivery, exerting a crucial effect in apoptosis. Therefore, inducing apoptosis through mitochondrial damage presents a viable avenue for tumor treatment.

Cyanine chromophores (Cy7-Cl), a derivative of heptamethine cyanine dyes developed and synthesized by our research groups, demonstrate anti-tumor effects through its chemotherapy activity (Figure 5A). On localization to mitochondria, Cy7-Cl induces mitochondrial dysfunction, influencing the expression of BAX and Bcl-2 through AMPK signaling. This process leads to a reduction in mitochondrial membrane potential and ATP content. Furthermore, Cy7-Cl affects the release of free Cyt C from mitochondria to the cytoplasm, thereby increasing ROS content and stimulating the activity of cleaved-caspase 3, ultimately inducing apoptosis in CRC cells.

Mitochondria are central organelles in the intrinsic apoptosis pathway of 2,2'- and 4,4'- cyanines (Figure 5B). Kadigamuwa *et al.* have demonstrated that these compounds can accumulate in mitochondria at high concentrations, resulting in mitochondrial membrane depolarization, ROS production, and apoptosis of cancer cells[194].

Yang *et al.* found that D112 (Figure 5C) induces caspase activation, mitochondrial depolarization, phosphatidylserine externalization, and Cyto C release, consequently activating caspase-9 and eventually inducing cell apoptosis. High doses of D112 trigger the mitochondrial apoptosis pathway, while low doses induce apoptosis and cell cycle arrest dependent on mitochondrial dysfunction. These results suggest that D112 is a potential small molecule warranting further investigation[195].

Patil *et al.* synthesized cyanine-based 3-methoxy pyrrole and other cyanine derivatives, including 7n (Figure 5D) and 7p (Figure 5E), which localize to the mitochondria of HeLa cells and induce cell damage. These small molecules interfere with anti-apoptosis (Bcl-2/Bcl-xl) and pro-apoptosis (BAX) proteins, produce ROS, arrest the cell cycle in the G0/G1 phase, activate caspase-3/9, and ultimately induce apoptosis of cervical cancer cells[46].

Chitooligosaccharides (COS) found in shrimp and crab shells exhibit promising anti-tumor activity and hold potential as adjuvant therapy alongside other chemotherapy drugs[196]. COS-Cy7, synthesized by Zhai *et al.*, has demonstrated

anti-tumor effects *in vitro*. It influences the proliferation, apoptosis, and ROS production of renal cancer cells, leading to G2/M phase arrest, apoptosis, and mitochondrial damage in a ROS-dependent manner. These findings suggest that COS-Cy7 may emerge as a potential therapeutic agent for tumor therapy, particularly for renal cancer[197]. Therefore, COS-Cy7 can induce apoptosis in cancer cells through a ROS-dependent mechanism, thereby exerting its anti-tumor effects.

5.1.2. PTT activity

Phototherapy is a local treatment approach offering several advantages, including minimal invasiveness, precise temporal and spatial control, and low susceptibility to drug resistance[198]. Among the primary phototherapy methods, PTT and PDT have gained significant attention in cancer research.

Photodynamic therapy involves the use of photosensitizers and molecular oxygen under laser irradiation of specific wavelengths to produce ROS, which exert substantial toxicity against tumor cells. In addition, PDT can inhibit tumor angiogenesis and activate anti-tumor immunity, thereby indirectly impeding tumor growth and metastasis[199-201].

Liu *et al.* developed a novel cyanine photosensitizer, Cy-N-Rh, designed for PDT (Figure 6A). This photosensitizer demonstrates excellent mitochondrial co-localization ability and can induce apoptosis by producing intracellular ROS, exhibiting low dark toxicity, elevated phototoxicity, and excellent biocompatibility[202].

Zhao *et al.* synthesized CYBF2, a cyanine photosensitizer tailored for cancers PDT. Compared with traditional anthocyanin photosensitizers (Figure 6B), CYBF2 displays red light (660 nm) photosensitivity and enhanced photostability. By producing ROS in cell mitochondria, CYBF2 effectively triggers cancer cell apoptosis through mitochondrial damage[203].

The study conducted by Kulbacka *et al.* investigated the effects of four cyanine derivatives, namely KF-570, HM-118, FBF-749, and ER-139, on malignant adenocarcinoma cells. Their findings revealed that HM-118 and KF-570 could induce apoptosis, necrosis, and autophagy in malignant adenocarcinoma cells[204-206]. Specifically, HM-118 was observed to induce apoptosis and reduce clonogenic ability in these cells, while ER-139 produced ROS upon irradiation, causing oxidative damage in malignant adenocarcinoma cells. This oxidative stress may induce cancer death through apoptosis, necrosis, and autophagy[207].

Furthermore, cyanine derivative IR-775 has demonstrated anti-tumor effects. Waszkiewicz *et al.* investigated the impact of cyanine IR-775 and 2-methoxyestradiol on ovarian and breast cancer cells (MDA-MB-231 and SK-OV-3). Their research revealed that IR-775 and 2-methodestradiol induce cancer cell apoptosis through PDT, thus exhibiting anti-tumor properties[189].

In addition, Krejcir *et al.* identified a novel anticancer molecule, a pentamethinium salt derivative (salt 1) (Figure 6C). Their study demonstrated that PDT targeting the mitochondria of tumor cells using salt 1 can disrupt mitochondrial structure, reduce cell metabolism, induce autophagy, and ultimately trigger apoptosis in cancer cells[208,209].

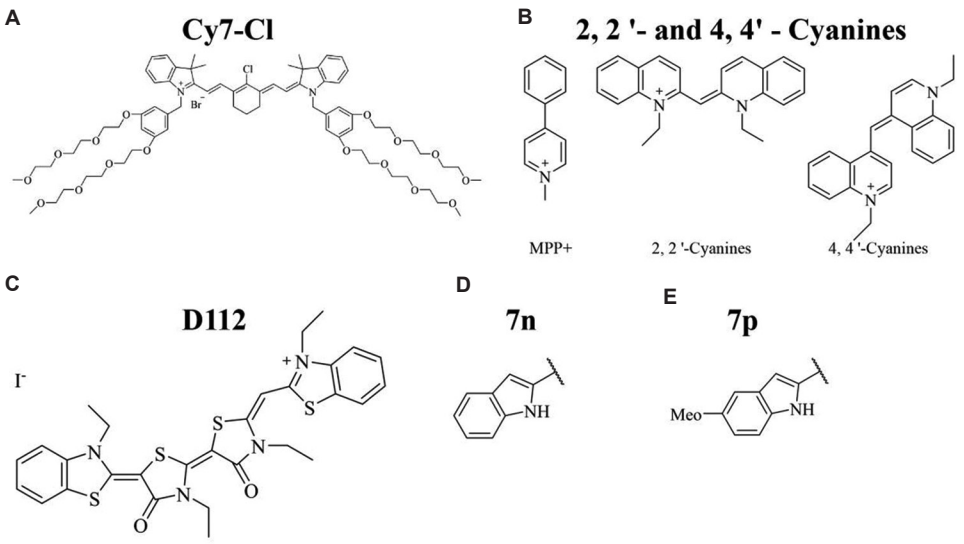

Figure 5. (A-E) Various cyanine compounds. These cyanine compounds can induce cancer cell apoptosis through chemotherapy, thus exerting anti-tumor activity.

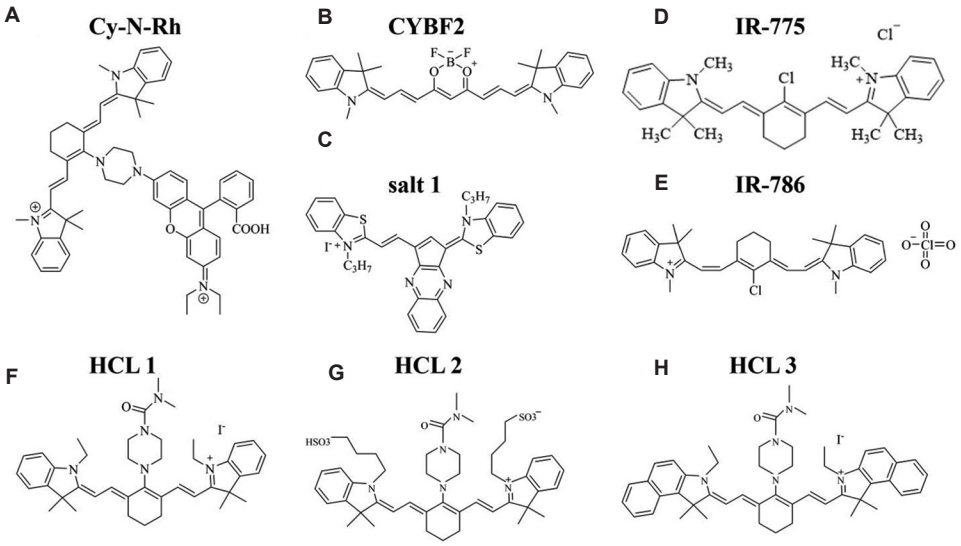

Figure 6. (A-H) Different small molecule cyanine compounds. These compounds can induce cancer cell apoptosis through PDT, thus exerting anti-tumor activity.

Wezgowiec *et al.* proposed the discovery of two cyanine compounds, namely IR-775 (Figure 6D) and IR-786 (Figure 6E), which have been demonstrated to act on MCF-7/WT cells through PDT without inducing any side effects on these cells. These compounds hold promise as antitumor agents capable of inducing cancer cell apoptosis through PDT[210]. In addition, IR-786 demonstrates potential as a molecular probe for fluorescent biological imaging[211].

Cai *et al.* designed and synthesized three lysosome-targeted fluorescent probes, namely, HCL 1 (Figure 6F), HCL 2 (Figure 6G), and HCL 3 (Figure 6H), based on the structure of Cy7. Lysosomes play a critical role in cell survival and apoptosis. On irradiation, both HCL 1 and HCL 3 are capable of producing ROS and inducing cell apoptosis. Meanwhile, HCL 2 exhibits effective tumor targeting and demonstrates a PDT effect *in vivo*[212].

PTT, which incorporates both PDT and PTT, has emerged as a tumor ablation method for treating various types of cancer. PDT can enhance the sensitivity of tumor cells to PTT by modulating the tumor microenvironment. In contrast, the heat produced by PTT can increase blood flow, enhance oxygen supply, and increase the therapeutic efficacy of PDT. Therefore, the combined therapy of PDT and PTT can induce apoptosis of cancer cells by producing high temperature or ROS, thereby exerting an anti-tumor effect[213].

IR-783, a NIF heptamethine cyanine dye with cancer-targeting properties, exhibits anticancer effects (Figure 7A). On one hand, as reported by Li *et al.*, IR-783 inhibits the proliferation and migration of breast cancer cells. It exerts a dose- and time-dependent inhibitory effect on MDA-MB-231 and MCF-7 cancer cells by inducing G0/G1 phase cell cycle arrest and reducing ATP levels. These findings indicate that IR-783 holds promise as a potential new drug for breast cancer treatment[214]. On the other hand, Tang *et al.* and Hou *et al.* observed that IR-783 reduces the activity of MDA-MB-231 cells and facilitates apoptosis in breast cancer cells by inducing mitochondrial fragmentation[215,216]. In addition, certain studies have demonstrated that IR-783 exhibits cancel cell uptake and accumulation, targeting brain, prostate, and colon tumors, with minimal toxicity to normal tissues[217-219]. Furthermore, IR-783 can reduce the activity of cancer cells through both PTT and PDT[220,221]. With its broad-spectrum anticancer activity and specificity for prostate, bladder, and breast cancer, IR-783 holds promise as a promising NIRF dye[222,223].

Cao *et al.* modified the indocyanine green derivative Cy7 with a heavy atom iodine to form a new NIR dye called CyI. Through PPT and PDT, CyI can rapidly and simultaneously generate ROS and heat, inducing increased cancer cell apoptosis and a higher inhibition rate in deep tumors. Importantly, while maintaining its fluorescence characteristics, CyI can be used for non-invasive *in vivo* imaging[188].

Sun *et al.* synthesized the near-infrared fluorescent small molecule compound IR-817 through the oxidation-reduction reaction of IR-808 and choline, providing an integrated approach to diagnosis and treatment (Figure 7B). IR-817 exhibits maximum absorption and emission peaks at 764 nm and 790 – 820 nm, respectively, within the NIR region (700 – 900 nm). The anti-tumor mechanism of IR-817 involves promoting the expression of BAX and inhibiting the expression of Bcl-2 protein, thereby increasing the BAX/Bcl-2 ratio and further triggering the

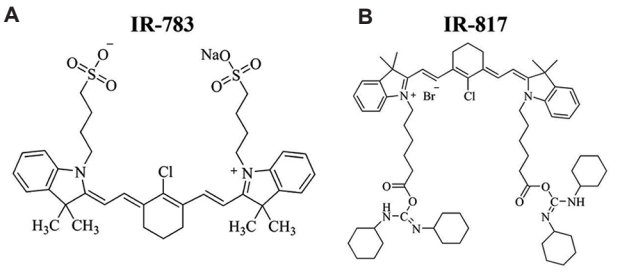

Figure 7. (A-B) Different small molecule cyanine compounds. These compounds can induce cancer cell apoptosis through PTT and PDT combined therapy, thus exerting anti-tumor activity.

caspase cascade reaction, leading to apoptosis of melanoma cells. In addition, IR-817 induces G0/G1 cell cycle arrest by targeting E2F/Cyclin/CDK signaling pathway without causing apparent toxicity and side effects. Therefore, this mitochondrial-targeted therapeutic drug, IR-817, holds promise for early diagnosis, real-time monitoring, and drug treatment of tumors[224].

5.2. Polymer nanoparticles

5.2.1. Chemotherapy activity

Encapsulating chemotherapy drugs in nanoparticles can reduce toxicity by enabling targeted delivery and controlled release[225].

In a study by Zhang *et al.*, a novel tumor-targeting nanomedicine named AS1411-T-5-FU was developed to enhance the therapeutic effectiveness against breast cancer. This nanomedicine incorporates Cy5, a fluorescent probe utilized for labeling DNA tetrahedrons, which improves their photostability without compromising their essential function. AS1411-T-5-FU offers many advantages, such as structural stability, biocompatibility, significant toxicity to cancer cells, and the ability to selectively target cancer cells. The mechanism of action of AS1411-T-5-FU involves inducing mitochondrial apoptosis, highlighting its potential as a promising novel anti-cancer drug with potent efficacy and selective toxicity on breast cancer cells[226].

5.2.2. Photothermal activity

Nanoparticle-based phototherapies, such as PTT and PDT, represent promising approaches for tumor eradication. These therapies not only directly target undetectable tumors and metastatic cancers but also hold the potential for treating other conditions, such as melanoma, and overcoming drug resistance. In addition, they can activate systemic immune responses by modulating the tumor microenvironment[227].

Photodynamic therapy exerts its anti-tumor effect by generating ROS and free radicals through interactions with the plasma nano-platform of the local electric field. These species exhibit cytotoxicity with a short half-life and low diffusion, ultimately leading to tumor cell apoptosis, autophagy, and necrosis[227].

In a study by Zhang *et al.*, folate (FA) and Cy7-modified chitosan (CF7) were chemically synthesized and developed into self-assembled nanoparticles (CF7Ns) for tumor-specific imaging and PDT. Experimental results demonstrated that CF7Ns induced apoptosis in HeLa cells on NIF light irradiation, thereby improving the therapeutic efficacy[228]. CF7Ns show significant promise as tumor-targeting agents[228].

Rizvi *et al.* designed Cy5.5-coupled self-assembled peptide nanoparticles (f-SAPNs), denoted as Cy5.5-c[RGD-KLAK]. Their study revealed that Cy5.5-c could produce ROS, disrupt mitochondrial membranes, activate caspase-3 enzymes, and induce apoptosis in cancer cells[229].

PTT and photodynamic therapy primarily utilize NIR and nanoparticles, serving as photosensitive and photothermal agents. In PTT, gold nanoparticles produce heat upon activation by specific wavelengths of light, effectively killing cancer cells. Similarly, PDT involves the production of ROS by gold nanoparticles to induce cancer cell death.

Han *et al.* developed Mito-Cy-Tfs, a mitochondrial-targeted fluorescence probe comprising three components: (i) NIF heptamethine cyanine as a fluorescence signal converter; (ii) trifluoromethyl sulfonamide as a fluorescence regulator; and (iii) lipophilic triphenylphosphonium cation as a mitochondrial guide. This cyanine derivative demonstrates apoptotic characteristics, such as alteration in the BAX/Bcl-2 ratio and the expression of Cyto C, cleaved-caspase-3, and cleaved-PARP[230].

Heptamethyl cyanine dye (HMCD) exhibits excellent tumor specificity and selectivity, capable of inducing apoptosis through mitochondrial damage, making it a promising candidate for treating brain tumors[231]. HMCD dyes are used for imaging due to their optimal NIF emission and excellent photophysical properties. Furthermore, they hold potential as effective drug carriers for brain tumor treatment (Figure 8).

HA-PEG-CyI (HPC) is novel therapeutic nano-carriers developed by Chi *et al.* Under 808 nm laser irradiation, HPC can produce ROS and elevate the temperature, triggering apoptosis and necrosis at the tumor site. HPC-induced cell death may initiate a range of acute inflammatory reactions, resulting in systemic immune induction and secondary death of tumor cells, further reducing tumor recurrence[232].

Yu *et al.* developed nano-ethanol loaded with IR-808 (IR-808-ES) as a novel nanoparticle-based photosensitizer for transdermal PDT/PTT of hypertrophic scars (HS).

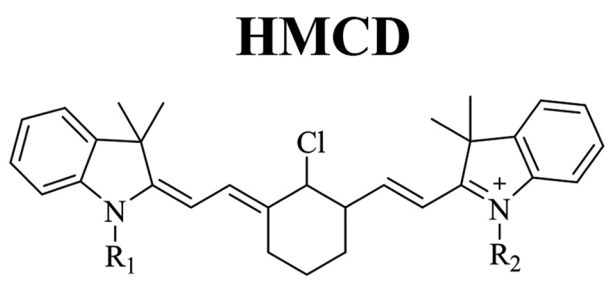

Figure 8. Cyanine polymer nanoparticle compounds. These compounds can induce cancer cell apoptosis through PDT and PTT, thus exerting anti-tumor activity.

In vivo experiments have validated that IR-808-ES enhances the efficacy of PDT/PTT treatment on HS by promoting apoptosis in HS tissue and facilitating the remodeling of collagen fibers. Consequently, IR-808-ES holds promising potential for clinical trials[233].

6. Conclusion

Cancer persists as the leading cause of mortality globally, presenting a substantial challenge to humanity and imposing a significant socioeconomic burden on countries. Despite the availability and significant progress in various cancer treatment strategies, including those approved for clinical use, chemotherapy continues to stand as the most widely recognized therapeutic measure. However, its utility is limited by a range of side effects, such as hepatotoxicity, cardiotoxicity, and nephrotoxicity[8]. The principal focus of these treatment strategies revolves around inducing the death of tumor cells, which can occur through various pathways, including apoptosis, necrosis, autophagy, ferroptosis, copper-induced cell death, and pyroptosis[208-210]. Recent endeavors in cancer treatment have underscored the significance of targeting apoptosis for the effective eradication of tumor cells[27]. Cyanine emerges as a promising candidate to fulfill this demand, offering several advantageous properties, such as favorable properties inherent to cyanine, excellent biocompatibility, and low toxicity. However, cyanine also presents certain limitations, notably the lower fluorescence quantum efficiency of cyanine dyes compared to other dyes, alongside the tendency for cyanine dyes with multichain structures to aggregate. Presently, numerous studies have elucidated the ability of cyanine and its derivatives to regulate apoptosis in tumor cells. However, the majority of these studies focus on cyanine-induced apoptosis through PPT and PDT, with limited studies on cyanine-induced tumor cell apoptosis through chemotherapy. Moreover, the direct targets of cyanine and its derivatives remain unclear in many studies, consequently impeding their translational utilization. Therefore, cyanine-induced tumor cell apoptosis remains a significant topic necessitating further exploration.

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Conflict of interest

The authors declare no conflicts of interest.

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References

1. Siegel RL, Miller KD, Jemal A, 2019, Cancer statistics, 2019. *CA Cancer J Clin*, 69(1): 7–34.

   https://doi.org/10.3322/caac.21551

2. Sung H, Siegel RL, Torre LA, *et al.*, 2019, Global Patterns in excess body weight and the associated cancer burden. *CA Cancer J Clin*, 69: 88–112.

   https://doi.org/10.3322/caac.21499

3. Das S, Shukla N, Singh SS, *et al.*, 2021, Mechanism of Interaction between autophagy and apoptosis in cancer. *Apoptosis*, 26: 512–533.

   https://doi.org/10.1007/s10495-021-01687-9

4. Liu JJ, Lin M, Yu JY, *et al.*, 2011, Targeting apoptotic and autophagic pathways for cancer therapeutics. *Cancer Lett*, 300: 105–114.

   https://doi.org/10.1016/j.canlet.2010.10.001

5. Chaudhry GE, Md Akim A, Sung YY, *et al.*, 2022, Cancer

and apoptosis: The apoptotic activity of plant and marine natural products and their potential as targeted cancer therapeutics. *Front Pharmacol*, 13: 842376.

https://doi.org/10.3389/fphar.2022.842376

6. Yuan L, Cai Y, Zhang L, *et al.*, 2021, Promoting apoptosis, a promising way to treat breast cancer with natural products: A comprehensive review. *Front Pharmacol*, 12: 801662.

https://doi.org/10.3389/fphar.2021.801662

7. Goldblatt ZE, Cirka HA, Billiar KL, 2021, Mechanical regulation of apoptosis in the cardiovascular system. *Ann Biomed Eng*, 49: 75–97.

https://doi.org/10.1007/s10439-020-02659-x

8. Ketelut-Carneiro N, Fitzgerald KA, 2022, Apoptosis, pyroptosis, and necroptosis-oh my! The many ways a cell can die. *J Mol Biol*, 434: 167378.

https://doi.org/10.1016/j.jmb.2021.167378

9. Pistritto G, Trisciuoglio D, Ceci C, *et al.*, 2016, Apoptosis as anticancer mechanism: Function and dysfunction of its modulators and targeted therapeutic strategies. *Aging* (*Albany NY*), 8: 603–619.

https://doi.org/10.18632/aging.100934

10. D'Arcy MS, 2019, Cell death: A review of the major forms of apoptosis, necrosis and autophagy. *Cell Biol Int*, 43: 582–592.

https://doi.org/10.1002/cbin.11137

11. Tao SC, Yuan T, Rui BY, *et al.*, 2017, Exosomes derived from human platelet-rich plasma prevent apoptosis induced by glucocorticoid-associated endoplasmic reticulum stress in rat osteonecrosis of the femoral head via the Akt/Bad/Bcl-2 signal pathway. *Theranostics*, 7: 733–750.

https://doi.org/10.7150/thno.17450

12. Sun L, Wang H, Wang Z, *et al.*, 2012, Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of Rip3 kinase. *Cell*, 148: 213–227.

https://doi.org/10.1016/j.cell.2011.11.031

13. Li X, Gong W, Wang H, *et al.*, 2019, O-GlcNAc transferase suppresses inflammation and necroptosis by targeting receptor-interacting serine/threonine-protein kinase 3. *Immunity*, 50: 576–590.e6.

https://doi.org/10.1016/j.immuni.2019.01.007

14. Santagostino SF, Assenmacher CA, Tarrant JC, *et al.*, 2021, Mechanisms of regulated cell death: Current perspectives. *Vet Pathol*, 58: 596–623.

https://doi.org/10.1177/03009858211005537

15. Proneth B, Conrad M, 2019, Ferroptosis and necroinflammation, a yet poorly explored link. *Cell Death Differ*, 26: 14–24.

https://doi.org/10.1038/s41418-018-0173-9

16. Conrad M, Angeli JPF, Vandenabeele P, *et al.*, 2016, Regulated necrosis: Disease relevance and therapeutic opportunities. *Nat Rev Drug Discov*, 15: 348–366.

https://doi.org/10.1038/nrd.2015.6

17. Li J, Cao, F, Yin HL, *et al.*, 2020, Ferroptosis: Past, present and future. *Cell Death Dis*, 11: 88.

https://doi.org/10.1038/s41419-020-2298-2

18. Yang F, He Y, Zhai Z, *et al.*, 2019, Programmed cell death pathways in the pathogenesis of systemic lupus erythematosus. *J Immunol Res*, 2019: 3638562.

https://doi.org/10.1155/2019/3638562

19. Cai J, Yi M, Tan Y, *et al.*, 2021, Natural product triptolide induces GSDME-mediated pyroptosis in head and neck cancer through suppressing mitochondrial hexokinase-II. *J Exp Clin Cancer Res*, 40: 190.

https://doi.org/10.1186/s13046-021-01995-7

20. Yu J, Li S, Qi J, *et al.*, 2019, Cleavage of GSDME by caspase-3 determines lobaplatin-induced pyroptosis in colon cancer cells. *Cell Death Dis*, 10: 193.

https://doi.org/10.1038/s41419-019-1441-4

21. Wu J, Lin S, Wan B, *et al.*, 2019, Pyroptosis in liver disease: New insights into disease mechanisms. *Aging Dis*, 10: 1094–1108.

https://doi.org/10.14336/AD.2019.0116

22. Roth KG, Mambetsariev I, Kulkarni P, *et al.*, 2020, The mitochondrion as an emerging therapeutic target in cancer. *Trends Mol Med*, 26: 119–134.

https://doi.org/10.1016/j.molmed.2019.06.009

23. Zaib S, Hayyat A, Ali N, *et al.*, 2022, Role of mitochondrial membrane potential and lactate dehydrogenase a in apoptosis. *Anticancer Agents Med Chem*, 22: 2048–2062.

https://doi.org/10.2174/1871520621666211126090906

24. Malcom J, Arnold O, Howlett JG, *et al.*, 2008, Canadian cardiovascular society consensus conference guidelines on heart failure--2008 update: Best practices for the transition of care of heart failure patients, and the recognition, investigation and treatment of cardiomyopathies. *Can J Cardiol*, 24: 21–40.

https://doi.org/10.1016/s0828-282x(08)70545-2

25. Shaddy RE, Olsen SL, Bristow MR, *et al.*, 1995, Efficacy and safety of metoprolol in the treatment of doxorubicin-induced cardiomyopathy in pediatric patients. *Am Heart J*, 129: 197–199.

https://doi.org/10.1016/0002-8703(95)90061-6

26. Hu Y, Shao Z, Cai X, *et al.*, 2019, Mitochondrial pathway is involved in advanced glycation end products-induced apoptosis of rabbit annulus fibrosus cells. *Spine* (*Phila Pa 1976*), 44: E585–E595.

https://doi.org/10.1097/BRS.0000000000002930

27. Carneiro BA, El-Deiry WS, 2020, Targeting apoptosis in cancer therapy. *Nat Rev Clin Oncol*, 17: 395–417.

https://doi.org/10.1038/s41571-020-0341-y

28. Boraldi F, Lofaro FD, Quaglino D, 2021, Apoptosis in the extraosseous calcification process. *Cells*. 10: 131.

https://doi.org/10.3390/cells10010131

29. Khodavirdipour A, Piri M, Jabbari S, *et al.*, 2021, Apoptosis detection methods in diagnosis of cancer and their potential role in treatment: Advantages and disadvantages: A review. *J gastrointest Cancer*, 52: 422–430.

https://doi.org/10.1007/s12029-020-00576-9

30. Wlodkowic D, Telford W, Skommer J, *et al.*, 2011, Apoptosis and beyond: Cytometry in Studies of programmed cell death. *Methods Cell Biol*, 103: 55–98.

https://doi.org/10.1016/B978-0-12-385493-3.00004-8

31. Cross CE, Halliwell B, Borish ET, *et al.*, 1987, Oxygen radicals and human disease. *Ann Intern Med*, 107: 526–545.

https://doi.org/10.7326/0003-4819-107-4-526

32. Liou GY, Storz P, 2010, Reactive oxygen species in cancer. *Free Radic Res*, 44: 479–496.

https://doi.org/10.3109/10715761003667554

33. Wenner CE, Spirtes MA, Weinhouse S, 1952, Metabolism of neoplastic tissue. II. A survey of enzymes of the citric acid cycle in transplanted tumors. *Cancer Res*, 12: 44–49.

34. Tsuchiya K, 2021, Switching from apoptosis to pyroptosis: Gasdermin-elicited inflammation and antitumor immunity. *Int J Mol Sci*, 22: 426.

https://doi.org/10.3390/ijms22010426

35. Hilska M, Collan YU, Laine VJ, *et al.*, 2005, The significance of tumor markers for proliferation and apoptosis in predicting survival in colorectal cancer. *Dis Colon Rectum*, 48: 2197–2208.

https://doi.org/10.1007/s10350-005-0202-x

36. Aaltomaa S, Kärjä V, Lipponen P, *et al.*, 2006, Expression of Ki-67, Cyclin D1 and apoptosis markers correlated with survival in prostate cancer patients treated by radical prostatectomy. *Anticancer Res,* 26: 4873–4878.

37. Wójcik P, Žarković N, Gęgotek A, *et al.*, 2020, Involvement of metabolic lipid mediators in the regulation of apoptosis. *Biomolecules*, 10: 402.

https://doi.org/10.3390/biom10030402

38. Dadsena S, Zollo C, García-Sáez AJ, 2021, Mechanisms of mitochondrial cell death. *Biochem Soc Trans*, 49: 663–674.

https://doi.org/10.1042/BST20200522

39. Tian C, Liu Y, Li Z, *et al.*, 2022, Mitochondria related cell death modalities and disease. *Front Cell Dev Biol*, 10: 832356.

https://doi.org/10.3389/fcell.2022.832356

40. Frezza C, Cipolat S, Martins de Brito O, *et al.*, 2006, OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell*, 126: 177–189.

https://doi.org/10.1016/j.cell.2006.06.025

41. Gupta S, Kass GE, Szegezdi E, *et al.*, 2009, The mitochondrial death pathway: A promising therapeutic target in diseases. *J Cell Mol Med,* 13: 1004–1033.

https://doi.org/10.1111/j.1582-4934.2009.00697.x

42. Calvo SE, Mootha VK, 2010, The mitochondrial proteome and human disease. *Ann Rev Genom Hum Genet,* 11: 25–44.

https://doi.org/10.1146/annurev-genom-082509-141720

43. Nunnari J, Suomalainen A, 2012, Mitochondria: In sickness and in health. *Cell*, 148: 1145–1159.

https://doi.org/10.1016/j.cell.2012.02.035

44. Gogvadze V, Orrenius S, Zhivotovsky B, 2008, Mitochondria in cancer cells: What Is so special about them? *Trends Cell Biol,* 18: 165–173.

https://doi.org/10.1016/j.tcb.2008.01.006

45. Boroughs LK, DeBerardinis RJ, 2015, Metabolic pathways promoting cancer cell survival and growth. *Nat Cell Biol*, 17: 351–359.

https://doi.org/10.1038/ncb3124

46. Patil S, Ghosh D, Radhakrishna M, *et al.*, 2020, Mitochondrial impairment by cyanine-based small molecules induces apoptosis in cancer cells. *ACS Med Chem Lett,* 11: 23–28.

https://doi.org/10.1021/acsmedchemlett.9b00304

47. Zhang L, Jia H, Liu X, *et al.*, 2021, Heptamethine cyanine-based application for cancer theranostics. *Front Pharmacol*, 12: 764654.

https://doi.org/10.3389/fphar.2021.764654

48. Wu XY, Xu WW, Huan XK, *et al.*, 2023, Najafi mechanisms of cancer cell killing by metformin: A review on different cell death pathways. *Mol Cell Biochem,* 478: 197–214.

https://doi.org/10.1007/s11010-022-04502-4

49. Tait SW, Green DR, 2010, Mitochondria and cell death: Outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol*, 11: 621–632.

https://doi.org/10.1038/nrm2952

50. Zhang Q, Liu J, Zhang M, *et al.*, 2019, Apoptosis induction of fibroblast-like synoviocytes is an important molecular-mechanism for herbal medicine along with its active components in treating rheumatoid arthritis. *Biomolecules*, 9: 795.

https://doi.org/10.3390/biom9120795

51. Gong YN, Crawford JC, Heckmann BL, *et al.*, 2019, To the edge of cell death and back. *FEBS J*, 286: 430–440.

https://doi.org/10.1111/febs.14714

52. Cain K, Brown DG, Langlais C, *et al.*, 1999, Caspase activation involves the formation of the aposome, a large (approximately 700 Kda) caspase-activating complex. *J Biol Chem,* 274: 22686–22692.

https://doi.org/10.1074/jbc.274.32.22686

53. Srinivasula SM, Ahmad M, Fernandes-Alnemri T, *et al.*, 1998, Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell,* 1: 949–957.

https://doi.org/10.1016/s1097-2765(00)80095-7

54. Li P, Nijhawan D, Budihardjo I, *et al.*, 1997, Cytochrome C and Datp-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. *Cell,* 91: 479–489.

https://doi.org/10.1016/s0092-8674(00)80434-1

55. Zou H, Henzel WJ, Liu X, *et al.*, 1997, Apaf-1, a human protein homologous to C. Elegans Ced-4, participates in cytochrome c-dependent activation of caspase-3. *Cell*, 90: 405–413.

https://doi.org/10.1016/s0092-8674(00)80501-2

56. Acehan D, Jiang X, Morgan DG, *et al.*, 2002, Three-Dimensional structure of the apoptosome: Implications for assembly, procaspase-9 binding, and activation. *Mol Cell,* 9: 423–432.

https://doi.org/10.1016/s1097-2765(02)00442-2

57. Kalkavan H, Green DR, 2018, Cell suicide as a Bcl-2 family business. *Cell Death Differ*, 25: 46–55.

https://doi.org/10.1038/cdd.2017.179

58. Ledgerwood EC, Morison IM, 2009, Targeting the apoptosome for cancer therapy. *Clin Cancer Res,* 15: 420–424.

https://doi.org/10.1158/1078-0432.CCR-08-1172

59. Liu X, Kim CN, Yang J, *et al.*, 1996, Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome C. *Cell,* 86: 147–157.

https://doi.org/10.1016/s0092-8674(00)80085-9

60. Bhola PD, Letai A, 2016, Mitochondria-judges and executioners of cell death sentences. *Mol Cell,* 61: 695–704.

https://doi.org/10.1016/j.molcel.2016.02.019

61. Jiang L, Zhang X, Zheng X, *et al.*, 2013, Apoptosis, senescence, and autophagy in rat nucleus pulposus cells: Implications for diabetic intervertebral disc degeneration. *J Orthop Res,* 31: 692–702.

https://doi.org/10.1002/jor.22289

62. Zhang F, Zhao X, Shen H, *et al.*, 2016, Molecular Mechanisms of cell death in intervertebral disc degeneration (review). *Int J Mol Med*, 37: 1439–1448.

https://doi.org/10.3892/ijmm.2016.2573

63. Green DR, Reed JC, 1998, Mitochondria and apoptosis. *Science* (*New York, NY*), 281: 1309–1312.

https://doi.org/10.1126/science.281.5381.1309

64. Kayacan S, Sener LT, Melikoglu G, *et al.*, 2018, Induction of apoptosis by *Centaurea nerimaniae* extract in HeLa and MDA-MB-231 cells by a caspase-3 pathway. *Biotech Histochem,* 93: 311–319.

https://doi.org/10.1080/10520295.2017.1401662

65. Nagata S, 2018, Apoptosis and clearance of apoptotic cells. *Annu Rev Immunol,* 36: 489–517.

https://doi.org/10.1146/annurev-immunol-042617-053010

66. Suzuki J, Denning DP, Imanishi E, *et al.*, 2013, Xk-related protein 8 and CED-8 promote phosphatidylserine exposure in apoptotic cells. *Science* (*New York, NY*), 341: 403–406.

https://doi.org/10.1126/science.1236758

67. Segawa K, Kurata S, Yanagihashi Y, *et al.*, 2014, Caspase-Mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. *Science* (*New York, NY*) 344: 1164–1168.

https://doi.org/10.1126/science.1252809

68. Suzuki J, Umeda M, Sims PJ, *et al.*, 2010, Calcium-dependent phospholipid scrambling by TMEM16F. *Nature*, 468: 834–838.

https://doi.org/10.1038/nature09583

69. Slee EA, Harte MT, Kluck RM, *et al.*, 1999, Ordering the cytochrome C-initiated caspase cascade: Hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol,* 144: 281–292.

https://doi.org/10.1083/jcb.144.2.281

70. Nicholson DW, Thornberry NA, 1997, Caspases: Killer proteases. *Trends Biochem Sci,* 22: 299–306.

https://doi.org/10.1016/s0968-0004(97)01085-2

71. Fraser A, Evan G, 1996, A license to kill. *Cell*, 85: 781–784.

https://doi.org/10.1016/s0092-8674(00)81005-3

72. Van de Craen M, Declercq W, Van den Brande I, *et al.*, 1999, The proteolytic procaspase activation network: An *in vitro* analysis. *Cell Death Differ*, 6: 1117–1124.

https://doi.org/10.1038/sj.cdd.4400589

73. Chandler JM, Cohen GM, MacFarlane M, 1998, Different subcellular distribution of caspase-3 and caspase-7 following FAS-induced apoptosis in mouse liver. *J Biol Chem,* 273: 10815–10818.

https://doi.org/10.1074/jbc.273.18.10815

74. Fan TJ, Han LH, Cong RS, *et al.*, 2005, Caspase family proteases and apoptosis. *Acta Biochim Biophys Sin* (Shanghai), 37: 719–727.

https://doi.org/10.1111/j.1745-7270.2005.00108.x

75. Wong RS, 2011, Apoptosis in cancer: From pathogenesis to treatment. *J Exp Clin Cancer Res,* 30: 87.

https://doi.org/10.1186/1756-9966-30-87

76. Kale J, Osterlund EJ, Andrews DW, 2018, Bcl-2 family proteins: Changing partners in the dance towards death. *Cell Death Differ,*25: 65–80.

https://doi.org/10.1038/cdd.2017.186

77. Yang R, Dong H, Jia S, 2022, Resveratrol as a modulatory of apoptosis and autophagy in cancer therapy. *Clin Transl Oncol*, 24: 1219–1230.

https://doi.org/10.1007/s12094-021-02770-y

78. Siddiqui WA, Ahad A, Ahsan H, 2015, The mystery of Bcl2 family: Bcl-2 proteins and apoptosis: An update. *Arch Toxicol,* 89: 289–317.

https://doi.org/10.1007/s00204-014-1448-7

79. Harada H, Grant S, 2003, Apoptosis regulators. *Rev Clin Exp Hematol,* 7: 117–138.

80. Wang K, Yin XM, Chao DT, *et al.*, 1996, Bid: A novel Bh3 domain-only death agonist. *Genes Dev,* 10: 2859–2869.

https://doi.org/10.1101/gad.10.22.2859

81. O'Connor L, Strasser A, O'Reilly LA, *et al.*, 1998, Bim: A novel member of the Bcl-2 family that promotes apoptosis. *EMBO J,* 17: 384–395.

https://doi.org/10.1093/emboj/17.2.384

82. Nakano K, Vousden KH, 2001, PUMA, a novel proapoptotic gene, is induced by P53. *Mol Cell,* 7: 683–694.

https://doi.org/10.1016/s1097-2765(01)00214-3

83. Cory S, Huang DC, Adams JM, 2003, The Bcl-2 family: Roles in cell survival and oncogenesis. *Oncogene*, 22: 8590–8607.

https://doi.org/10.1038/sj.onc.1207102

84. Gross A, McDonnell JM, Korsmeyer SJ, 1999, Bcl-2 family members and the mitochondria in apoptosis. *Genes Dev*, 13: 1899–1911.

https://doi.org/10.1101/gad.13.15.1899

85. Shakeri R, Kheirollahi A, Davoodi J, 2017, Apaf-1: Regulation and function in cell death. *Biochimie*, 135: 111–125.

https://doi.org/10.1016/j.biochi.2017.02.001

86. Bruckheimer EM, Cho SH, Sarkiss M, *et al.*, 1998, The Bcl-2 gene family and apoptosis. *Adv Biochem Eng Biotechnol,* 62: 75–105.

https://doi.org/10.1007/BFb0102306

87. Gloire G, Charlier E, Piette J, 2008, Regulation of Cd95/Apo-1/Fas-induced apoptosis by protein phosphatases. *Biochem Pharmacol,* 76: 1451–1458.

https://doi.org/10.1016/j.bcp.2008.06.023

88. Gottlieb TM, Oren M, 1998, P53 and apoptosis. *Semin Cancer Biol*, 8: 359–368.

https://doi.org/10.1006/scbi.1998.0098

89. McMahon SB, 2014, Myc and the control of apoptosis. *Cold Spring Harb Perspect Med*, 4: a014407.

https://doi.org/10.1101/cshperspect.a014407

90. Smith GC, d'Adda di Fagagna F, Lakin ND, 1999, Cleavage and inactivation of Atm during apoptosis. *Mol Cell Biol,* 19: 6076–6084.

https://doi.org/10.1128/mcb.19.9.6076

91. Bleicken S, Jeschke G, Stegmueller C, *et al.*, 2014, Structural model of active bax at the membrane. *Mol Cell,* 56: 496–505.

https://doi.org/10.1016/j.molcel.2014.09.022

92. Borner C, Andrews DW, 2014, The apoptotic pore on mitochondria: Are we breaking through or still stuck? *Cell Death Differ,* 21: 187–191.

https://doi.org/10.1038/cdd.2013.169

93. Jeong SH, Lee HW, Han JH, *et al.*, 2008, Low expression of bax predicts poor prognosis in resected non-small cell lung cancer patients with non-squamous histology. *Jpn J Clin Oncol,* 38: 661–669.

https://doi.org/10.1093/jjco/hyn089

94. Schuyer M, van der Burg ME, Henzen-Logmans SC, *et al.*, 2001, Reduced expression of bax is associated with poor prognosis in patients with epithelial ovarian cancer: A multifactorial analysis of Tp53, P21, Bax and Bcl-2. *Br J Cancer,* 85: 1359–1367.

https://doi.org/10.1054/bjoc.2001.2101

95. Indran IR, Tufo G, Pervaiz S, *et al.*, 2011, Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. *Biochim Biophys Acta,* 1807: 735–745.

https://doi.org/10.1054/bjoc.2001.2101

96. Miquel C, Borrini F, Grandjouan S, *et al.*, 2005, Role of bax mutations in apoptosis in colorectal cancers with microsatellite instability. *Am J Clin Pathol,* 123: 562–570.

https://doi.org/10.1309/JQ2X-3RV3-L8F9-TGYW

97. Pepper C, Hoy T, Bentley DP, 1997, Bcl-2/Bax ratios in chronic lymphocytic leukaemia and their correlation with *in vitro* apoptosis and clinical resistance. *Br J Cancer,* 76: 935–938.

https://doi.org/10.1038/bjc.1997.487

98. Soengas MS, Capodieci P, Polsky D, *et al.*, 2001, Lowe inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature,* 409: 207–211.

https://doi.org/10.1038/35051606

99. Baldi A, Santini D, Russo P, *et al.*, 2004, Analysis of Apaf-1 expression in human cutaneous melanoma progression. *Exp Dermatol,* 13: 93–97.

https://doi.org/10.1111/j.0906-6705.2004.00136.x

100. Wan J, Kalpage HA, Vaishnav A, *et al.*, 2019, Regulation of respiration and apoptosis by cytochrome C threonine 58 phosphorylation. *Sci Rep,* 9: 15815.

https://doi.org/10.1038/s41598-019-52101-z

101. Kagan VE, Bayir HA, Belikova NA, *et al.*, 2009, Cytochrome C/cardiolipin relations in mitochondria: A kiss of death. *Free Radic Biol Med,* 46: 1439–1453.

https://doi.org/10.1016/j.freeradbiomed.2009.03.004

102. Kagan VE, Tyurin VA, Jiang J, *et al.*, 2005, Cytochrome C acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat Chem Biol,* 1: 223–232.

https://doi.org/10.1038/nchembio727

103. Vladimirov YA, Proskurnina EV, Izmailov DY, *et al.*, 2006, Cardiolipin activates cytochrome C peroxidase activity since it facilitates H(2)O(2) access to heme. *Biochemistry* (*Mosc*), 71: 998–1005.

https://doi.org/10.1134/s0006297906090082

104. Thornberry NA, Lazebnik Y, 1998, Caspases: Enemies within. *Science* (*New York, NY*), 281: 1312–1316.

https://doi.org/10.1126/science.281.5381.1312

105. Nicholson DW, Ali A, Thornberry NA, *et al.*, 1995, Identification and inhibition of the Ice/Ced-3 protease necessary for mammalian apoptosis. *Nature,* 376: 37–43.

https://doi.org/10.1038/376037a0

106. Fernandes-Alnemri T, Litwack G, Alnemri ES, 1994, Cpp32, a novel human apoptotic protein with homology to caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. *J Biol Chem,* 269: 30761–30764.

https://doi.org/10.1016/S0021-9258(18)47344-9

107. Cohen GM, 1997, Caspases: The executioners of apoptosis. *Biochem J,* 326 (Pt 1): 1–16.

https://doi.org/10.1042/bj3260001

108. Medina V, Edmonds B, Young GP, *et al.*, 1997, Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin a (inhibitors of histone deacetylase): Dependence on protein synthesis and synergy with a mitochondrial/cytochrome C-dependent pathway. *Cancer Res,* 57: 3697–3707.

109. Asselin E, Mills GB, Tsang BK, 2001, XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. *Cancer Res,* 61: 1862–1868.

110. Törmänen-Näpänkangas U, Soini Y, Kahlos K, *et al.*, 2001, Expression of Caspases-3, -6 and -8 and their relation to apoptosis in non-small cell lung carcinoma. *Int J Cancer* 93: 192–198.

https://doi.org/10.1002/ijc.1315

111. Hsia JY, Chen CY, Chen JT, *et al.*, 2003, Prognostic significance of caspase-3 expression in primary resected esophageal squamous cell carcinoma. *Eur J Surg Oncol,* 29: 44–48.

https://doi.org/10.1053/ejso.2002.1338

112. Kania J, Konturek SJ, Marlicz K, *et al.*, 2003, Expression of Survivin and caspase-3 in gastric cancer. *Digest Dis Sci,* 48: 266–271.

https://doi.org/10.1023/a:1021915124064

113. Li Y, Yu H, Zhao L, *et al.*, 2020, Effects of carbon nanotube-mediated caspase3 gene silencing on cardiomyocyte apoptosis and cardiac function during early acute myocardial infarction. *Nanoscale,* 12: 21599–21604.

https://doi.org/10.1039/D0NR05032F

114. Vaux DL, Cory S, Adams JM, 1988, Bcl-2 gene promotes haemopoietic cell survival and cooperates with C-Myc to immortalize pre-B cells. *Nature*, 335: 440–442.

https://doi.org/10.1038/335440a0

115. Šošić D, Richardson JA, Yu K, *et al.*, 2003, Twist regulates cytokine gene expression through a negative feedback loop that represses Nf-Kappab activity. *Cell,* 112: 169–180.

https://doi.org/10.1016/s0092-8674(03)00002-3

116. Liu JR, Chen BQ, Yang YM, *et al.*, 2002, Effect of apoptosis on gastric adenocarcinoma cell line Sgc-7901 induced by Cis-9, Trans-11-conjugated linoleic acid. *World J Gastroenterol*, 8: 999–1004.

https://doi.org/10.3748/wjg.v8.i6.999

117. Zhao AG, Zhao HL, Jin XJ, *et al.*, 2002, Effects of Chinese Jianpi herbs on cell apoptosis and related gene expression in human gastric cancer grafted onto nude mice. *World J Gastroenterol,* 8: 792–796.

https://doi.org/10.3748/wjg.v8.i5.792

118. Wu YL, Sun B, Zhang XJ, *et al.*, 2001, Growth inhibition and apoptosis induction of sulindac on human gastric cancer cells. *World J Gastroenterol,* 7: 796–800.

https://doi.org/10.3748/wjg.v7.i6.796

119. Siervo-Sassi RR, Marrangoni AM, Feng X, *et al.*, 2003, Physiological and molecular effects of Apo2l/trail and cisplatin in ovarian carcinoma cell lines. *Cancer Lett,* 190: 61–72.

https://doi.org/10.1016/s0304-3835(02)00579-7

120. Rudin CM, Yang Z, Schumaker LM, *et al.*, 2003, Cullen inhibition of glutathione synthesis reverses Bcl-2-mediated cisplatin resistance. *Cancer Res,* 63: 312–318.

121. Chen J, 2016, The cell-cycle arrest and apoptotic functions of P53 in tumor initiation and progression. *Cold Spring Harb Perspect Med,* 6: a026104.

https://doi.org/10.1101/cshperspect.a026104

122. Edison N, Zuri D, Maniv I, *et al.*, 2012, The Iap-antagonist arts initiates caspase activation upstream of cytochrome C and Smac/Diablo. *Cell Death Differ,* 19: 356–368.

https://doi.org/10.1038/cdd.2011.112

123. Raffo AJ, Perlman H, Chen MW, *et al.*, 1995, Overexpression of Bcl-2 protects prostate cancer cells from apoptosis *in vitro* and confers resistance to androgen depletion *in vivo*. *Cancer Res,* 55: 4438–4445.

124. Fulda S, Meyer E, Debatin KM, 2002, Inhibition of trail-induced apoptosis by Bcl-2 overexpression. *Oncogene,* 21: 2283–2294.

https://doi.org/10.1038/sj.onc.1205258

125. Foreman KE, Wrone-Smith T, Boise LH, *et al.*, 1996, Kaposi's sarcoma tumor cells preferentially express Bcl-Xl. *Am J Pathol,* 149: 795–803.

126. Krajewska M, Moss SF, Krajewski S, *et al.*, 1996, Elevated expression of Bcl-X and reduced bak in primary colorectal adenocarcinomas. *Cancer Res,* 56: 2422–2427.

127. Han JY, Hong EK, Choi BG, *et al.*, 2003, Death receptor 5 and Bcl-2 protein expression as predictors of tumor response to gemcitabine and cisplatin in patients with advanced non-small-cell lung cancer. *Med Oncol* (*Northwood, London, England*), 20: 355–362.

https://doi.org/10.1385/MO:20:4:355

128. Erovic BM, Pelzmann M, Grasl M, *et al.*, 2005, Mcl-1, vascular endothelial growth factor-R2, and 14-3-3sigma expression might predict primary response against radiotherapy and chemotherapy in patients with locally advanced squamous cell carcinomas of the head and neck. *Clin Cancer Res,* 11: 8632–8636.

https://doi.org/10.1158/1078-0432.CCR-05-1170

129. Williams J, Lucas PC, Griffith KA, *et al.*, 2005, Expression of Bcl-Xl in ovarian carcinoma is associated with chemoresistance and recurrent disease. *Gynecol Oncol,* 96: 287–295.

https://doi.org/10.1016/j.ygyno.2004.10.026

130. Michaud WA, Nichols AC, Mroz EA, *et al.*, 2009, Bcl-2 blocks cisplatin-induced apoptosis and predicts poor outcome following chemoradiation treatment in advanced oropharyngeal squamous cell carcinoma. *Clin Cancer Res,* 15: 1645–1654.

https://doi.org/10.1158/1078-0432.CCR-08-2581

131. Yang D, Chen MB, Wang LQ, *et al.*, 2013, Bcl-2 expression predicts sensitivity to chemotherapy in breast cancer: A systematic review and meta-analysis. *J Exp Clin Cancer Res,* 32: 105.

https://doi.org/10.1186/1756-9966-32-105

132. Edison N, Curtz Y, Paland N, *et al.*, 2017, Degradation of Bcl-2 by Xiap and Arts promotes apoptosis. *Cell Rep,* 21: 442–454.

https://doi.org/10.1016/j.celrep.2017.09.052

133. Wang X, Simpson ER, Brown KA, 2015, P53: Protection against tumor growth beyond effects on cell cycle and apoptosis. *Cancer Res,* 75: 5001–5007.

https://doi.org/10.1158/0008-5472.CAN-15-0563

134. Yu G, Luo H, Zhang N, *et al.*, 2019, Loss of P53 sensitizes cells to palmitic acid-induced apoptosis by reactive oxygen species accumulation. *Int J Mol Sci,* 20: 6268.

https://doi.org/10.3390/ijms20246268

135. Vousden KH, 2000, P53: Death star. *Cell,* 103: 691–694.

https://doi.org/10.1016/s0092-8674(00)00171-9

136. Chi SW, 2014, Structural insights into the transcription-independent apoptotic pathway of P53. *BMB Rep,* 47: 167–172.

https://doi.org/10.5483/BMBRep.2014.47.3.261

137. Tomita Y, Marchenko N, Erster S, *et al.*, 2006, WT P53, but not tumor-derived mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization. *J Biol Chem,* 281: 8600–8606.

https://doi.org/10.1074/jbc.M507611200

138. Marchenko ND, Moll UM, 2014, Mitochondrial death functions of P53. *Mol Cell Oncol,* 1: e955995.

https://doi.org/10.1080/23723548.2014.955995

139. Moll UM, Zaika A, 2001, Nuclear and mitochondrial apoptotic pathways of P53. *FEBS Lett,* 493: 65–69.

https://doi.org/10.1016/s0014-5793(01)02284-0

140. Mihara M, Erster S, Zaika A, *et al.*, 2003, P53 Has a direct apoptogenic role at the mitochondria. *Mol Cell,* 11: 577–590.

https://doi.org/10.1016/s1097-2765(03)00050-9

141. Aubrey BJ, Kelly GL, Janic A, *et al.*, 2018, How does P53 induce apoptosis and how does this relate to P53-mediated tumour suppression? *Cell Death Differ,* 25: 104–113.

https://doi.org/10.1038/cdd.2017.169

142. Vogelstein B, Lane D, Levine AJ, 2000, Surfing the P53 network. *Nature,* 408: 307–310.

https://doi.org/10.1038/35042675

143. Donepudi M, Grütter MG, 2002, Structure and zymogen activation of caspases. *Biophys Chem,* 101–102: 145–153.

https://doi.org/10.1016/s0301-4622(02)00151-5

144. O'Connor L, Harris AW, Strasser A, 2000, Cd95 (Fas/Apo-1) and P53 signal apoptosis independently in diverse cell types. *Cancer Res,* 60: 1217–1220.

145. Liu X, Yue P, Khuri FR, *et al.*, 2004, P53 upregulates death receptor 4 expression through an intronic P53 binding

site. *Cancer Res,* 64: 5078–5083.

https://doi.org/10.1158/0008-5472.CAN-04-1195

146. Oda E, Ohki R, Murasawa H, *et al.*, 2000, Noxa a Bh3-only member of the Bcl-2 family and candidate mediator of P53-induced apoptosis. *Science* (*New York, NY*), 288: 1053–1058.

https://doi.org/10.1126/science.288.5468.1053

147. Fridman JS, Lowe SW, 2003, Control of apoptosis by P53. *Oncogene,* 22: 9030–9040.

https://doi.org/10.1038/sj.onc.1207116

148. Hao Q, Chen J, Liao J, *et al.*, 2021, P53 induces arts to promote mitochondrial apoptosis. *Cell Death Dis,* 12: 204.

https://doi.org/10.1038/s41419-021-03463-8

149. Liu T, Wang L, Chen H, *et al.*, 2017, Molecular and cellular mechanisms of apoptosis during dissociated spermatogenesis. *Front Physiol*, 8: 188.

https://doi.org/10.3389/fphys.2017.00188

150. Hancock JF, 2003, Ras proteins: Different signals from different locations. *Nat Rev Mol Cell Biol,* 4: 373–384.

https://doi.org/10.1038/nrm1105

151. Alzahrani AS, 2019, Pi3k/Akt/Mtor inhibitors in cancer: At the bench and bedside. *Semin Cancer Biol,* 59: 125–132.

https://doi.org/10.1016/j.semcancer.2019.07.009

152. Carnero A, Blanco-Aparicio C, Renner O, *et al.*, 2008, Leal The Pten/Pi3k/Akt signalling pathway in cancer, therapeutic implications. *Curr Cancer Drug Targets,* 8: 187–198.

https://doi.org/10.2174/156800908784293659

153. Manning BD, Toker A, 2017, Akt/Pkb signaling: Navigating the network. *Cell,* 169: 381–405.

https://doi.org/10.1016/j.cell.2017.04.001

154. Dummler B, Hemmings BA, 2007, Physiological roles of Pkb/Akt isoforms in development and disease. *Biochem Soc Trans,* 35: 231–235.

https://doi.org/10.1042/BST0350231

155. Szymonowicz K, Oeck S, Malewicz NM, *et al.*, 2018, New Insights into protein kinase B/Akt signaling: Role of localized Akt Activation and compartment-specific target proteins for the cellular radiation response. *Cancers,* 10: 78.

https://doi.org/10.3390/cancers10030078

156. Revathidevi S, Munirajan AK, 2019, Akt in Cancer: Mediator and more. *Semin Cancer Biol,* 59: 80–91.

https://doi.org/10.1016/j.semcancer.2019.06.002

157. Risso G, Blaustein M, Pozzi B, *et al.*, 2015, Akt/Pkb: One kinase, many modifications. *Biochem J,* 468: 203–214.

https://doi.org/10.1042/BJ20150041

158. Reyes-Farias M, Carrasco-Pozo C, 2019, The anti-cancer effect of quercetin: Molecular implications in cancer metabolism. *Int J Mol Sci,* 20: 3177.

https://doi.org/10.3390/ijms20133177

159. Liu R, Chen Y, Liu G, *et al.*, 2020, Pi3k/Akt pathway as a key link modulates the multidrug resistance of cancers. *Cell Death Dis,* 11: 797.

https://doi.org/10.1038/s41419-020-02998-6

160. Sochalska M, Tuzlak S, Egle A, *et al.*, 2015, Lessons from gain- and loss-of-function models of pro-survival Bcl2 family proteins: Implications for targeted therapy. *FEBS J,* 282: 834–849.

https://doi.org/10.1111/febs.13188

161. Choi E, Kim E, Kim JH, *et al.*, 2019, Akt1-targeted proapoptotic activity of compound K in human breast cancer cells. *J Ginseng Res,* 43: 692–698.

https:/doi.org/10.1016/j.jgr.2019.07.001

162. Adams JM, Cory S, 2018, The Bcl-2 arbiters of apoptosis and their growing role as cancer targets. *Cell Death Differ,* 25: 27–36.

https://doi.org/10.1038/cdd.2017.161

163. Chen YH, Yang SF, Yang CK, *et al.*, 2021, Metformin induces apoptosis and inhibits migration by activating the Ampk/P53 axis and suppressing Pi3k/Akt signaling in human cervical cancer cells. *Mol Med Rep,* 23: 88.

https://doi.org/10.3892/mmr.2020.11725

164. Viollet B, Horman S, Leclerc J, *et al.*, 2010, AMPK inhibition in health and disease. *Crit Rev Biochem Mol Biol,* 45: 276–295.

https://doi.org/10.3109/10409238.2010.488215

165. Gui D, Cui Z, Zhang L, *et al.*, 2017, Salidroside Attenuates hypoxia-induced pulmonary arterial smooth muscle cell proliferation and apoptosis resistance by upregulating autophagy through the Ampk-Mtor-Ulk1 pathway. *BMC Pulm Med,* 17: 191.

https://doi.org/10.1186/s12890-017-0477-4

166. White JP, Billin AN, Campbell ME, *et al.*, 2018, The Ampk/P27(Kip1) axis regulates autophagy/apoptosis decisions in aged skeletal muscle stem cells. *Stem Cell Rep,* 11: 425–439.

https://doi.org/10.1016/j.stemcr.2018.06.014

167. Nitulescu GM, Van De Venter M, Nitulescu G, *et al.*, 2018, The Akt pathway in oncology therapy and beyond (review). *Int J Oncol,* 53: 2319–2331.

https://doi.org/10.3892/ijo.2018.4597

168. Cha JH, Yang WH, Xia W, *et al.*, 2018, Metformin promotes antitumor immunity via endoplasmic-

reticulum-associated degradation of Pd-L1. *Mol Cell,* 71: 606–620.e607.

https://doi.org/10.3892/ijo.2018.4597

169. Deng M, Yang X, Qin B, *et al.*, 2016, Deubiquitination and activation of Ampk by Usp10. *Mol Cell,* 61: 614–624.

https://doi.org/10.1016/j.molcel.2016.01.010

170. Faubert B, Vincent EE, Poffenberger MC, 2015, The Amp-activated protein kinase (Ampk) and cancer: Many faces of a metabolic regulator. *Cancer Lett,* 356: 165–170.

https://doi.org/10.1074/jbc.RA119.010243

171. Roberts DJ, Miyamoto S, 2015, Hexokinase Ii integrates energy metabolism and cellular protection: Akting on mitochondria and torcing to autophagy. *Cell Death Differ,* 22: 248–257.

https://doi.org/10.1038/cdd.2014.173

172. Laplante M, Sabatini DM, 2012, Mtor signaling in growth control and disease. *Cell,* 149: 274–293.

https://doi.org/10.1016/j.cell.2012.03.017

173. Lien EC, Dibble CC, Toker A, 2017, Pi3k signaling in cancer: Beyond Akt. *Curr Opin Cell Biol,* 45: 62–71.

https://doi.org/10.1016/j.ceb.2017.02.007

174. Lien EC, Lyssiotis CA, Cantley LC, 2016, Metabolic reprogramming by the Pi3k-Akt-Mtor pathway in cancer. *Recent Results Cancer Res,* 207: 39–72.

https://doi.org/10.1007/978-3-319-42118-6_3

175. Sui X, Kong N, Ye L, *et al.*, 2014, P38 and Jnk Mapk pathways control the balance of apoptosis and autophagy in response to chemotherapeutic agents. *Cancer Lett,* 344: 174–179.

https://doi.org/10.1016/j.canlet.2013.11.019

176. Chen M, Sun J, Lu C, *et al.*, 2016, The impact of neuronal Notch-1/Jnk pathway on intracerebral hemorrhage-induced neuronal injury of rat model. *Oncotarget,* 7: 73903–73911.

https://doi.org/10.18632/oncotarget.12094

177. Musi CA, Agrò G, Santarella F, *et al.*, 2020, Jnk3 as Therapeutic target and biomarker in neurodegenerative and neurodevelopmental brain diseases. *Cells,* 9: 2190.

https://doi.org/10.3390/cells9102190

178. Tan J, Gao W, Yang W, *et al.*, 2021, Isoform-specific functions of C-Jun N-terminal kinase 1 and 2 in lung ischemia-reperfusion injury through the C-Jun/activator protein-1 pathway. *J Thorac Cardiovasc Surg,* 162: e143–e156.

https://doi.org/10.1016/j.jtcvs.2020.03.083

179. Wang H, Zhong L, Mi S, *et al.*, 2020, Tanshinone Iia prevents platelet activation and down-regulates Cd36 and Mkk4/Jnk2 signaling pathway. *BMC Cardiovasc Disord,* 20: 81.

https://doi.org/10.1186/s12872-019-01289-z

180. Desai S, Laskar S, Pandey BN, 2013, Autocrine Il-8 and Vegf mediate epithelial-mesenchymal transition and invasiveness via P38/Jnk-Atf-2 signalling in A549 lung cancer cells. *Cell Signal,* 25: 1780–1791.

https://doi.org/10.1016/j.cellsig.2013.05.025

181. Ngoei KR, Catimel B, Church N, *et al.*, 2011, Characterization of a Novel Jnk (C-Jun N-terminal kinase) inhibitory peptide. *Biochem J,* 434: 399–413.

https://doi.org/10.1042/BJ20101244

182. Matsui Y, Kuwabara T, Eguchi T, *et al.*, 2018, Acetylation regulates the Mkk4-Jnk pathway in T cell receptor signaling. *Immunol Lett,* 194: 21–28.

https://doi.org/10.1016/j.imlet.2017.12.00

183. Paul M, Hemshekhar M, Thushara RM, *et al.*, 2015, Methotrexate promotes platelet apoptosis via Jnk-mediated mitochondrial damage: Alleviation by N-acetylcysteine and N-acetylcysteine amide. *PLoS One,* 10: e0127558.

https://doi.org/10.1038/sj.onc.1205258

184. Li Y, Zhou Y, Yue X, *et al.*, 2021, Cyanine conjugates in cancer theranostics. *Bioact Mater,* 6: 794–809.

https://doi.org/10.1016/j.bioactmat.2020.09.009

185. Fang M, Xia S, Bi J, *et al.*, 2018, A Cyanine-based fluorescent cassette with aggregation-induced emission for sensitive detection of Ph changes in live cells. *Chem Commun* (*Cambridge, England*), 54: 1133–1136.

https://doi.org/10.1039/C7CC08986D

186. Gui L, Yuan Z, Kassaye H, *et al.*, 2018, A Tumor-targeting probe based on a mitophagy process for live imaging. *Chem Commun* (*Cambridge, England*), 54: 9675–9678.

https://doi.org/10.1039/C8CC04246B

187. Liu R, Tang J, Xu Y, *et al.*, 2019, Bioluminescence imaging of inflammation *in vivo* based on bioluminescence and fluorescence resonance energy transfer using nanobubble ultrasound contrast agent. *ACS Nano,* 13: 5124–5132.

https://doi.org/10.1021/acsnano.8b08359

188. Cao J, Chi J, Xia J, *et al.*, 2019, Iodinated cyanine dyes for fast near-infrared-guided deep tissue synergistic phototherapy. *ACS Appl Mater Interfaces,* 11: 25720–25729.

https://doi.org/10.1021/acsami.9b07694

189. Waszkiewicz M, Choromanska A, Kulbacka J, *et al.*, 2022, The photodynamic reaction with Ir-775 cyanine

combined with 2-methoxyestradiol in ovarian (Skov-3) and human breast adenocarcinoma (Mda Mb-231) cell lines. *Photodiagn Photodyn Ther,* 38: 102766.

https://doi.org/10.1016/j.pdpdt.2022.102766

190. Choi SY, Baek SH, Chang SJ, *et al.*, 2017, Synthesis of upconversion nanoparticles conjugated with graphene oxide quantum dots and their use against cancer cell imaging and photodynamic therapy. *Biosens Bioelectron,* 93: 267–273.

https://doi.org/10.1016/j.bios.2016.08.094

191. Chudy M, Tokarska K, Jastrzębska E, *et al.*, 2018, Lab-on-a-chip systems for photodynamic therapy investigations. *Biosens Bioelectron,* 101: 37–51.

https://doi.org/10.1016/j.bios.2017.10.013

192. Shamjith S, Joseph MM, Murali VP, *et al.*, 2022, NADH-depletion triggered energy shutting with cyclometalated iridium (Iii) complex enabled bimodal luminescence-sers sensing and photodynamic therapy. *Biosens Bioelectron,* 204: 114087.

https://doi.org/10.1016/j.bios.2022.114087

193. Bilici K, Cetin S, Celikbas E, *et al.*, 2021, Recent advances in cyanine-based phototherapy agents. *Front Chem,* 9: 707876.

https://doi.org/10.3389/fchem.2021.707876

194. Kadigamuwa CC, Le VQ, Wimalasena K, 2015, 2, 2'- and 4, 4'-Cyanines are transporter-independent *in vitro* dopaminergic toxins with the specificity and mechanism of toxicity similar to Mpp+. *J Neurochem,* 135: 755–767.

https://doi.org/10.1111/jnc.13201

195. Yang N, Gilman P, Mirzayans R, *et al.*, 2015, Characterization of the apoptotic response induced by the cyanine dye D112: A potentially selective anti-cancer compound. *PLoS One,* 10: e0125381.

https://doi.org/10.1371/journal.pone.0125381

196. Zhai X, Yuan S, Yang X, *et al.*, 2018, Growth-inhibition of S180 residual-tumor by combination of cyclophosphamide and chitosan oligosaccharides *in vivo*. *Life Sci,* 202: 21–27.

https://doi.org/10.1016/j.lfs.2018.04.004

197. Zhai X, Yuan S, Yang X, *et al.*, 2019, Chitosan Oligosaccharides induce apoptosis in human renal carcinoma via reactive-oxygen-species-dependent endoplasmic reticulum stress. *J Agric Food Chem,* 67: 1691–1701.

https://doi.org/10.1021/acs.jafc.8b06941

198. Li X, Lovell JF, Yoon J, *et al.*, 2020, Clinical development and potential of photothermal and photodynamic therapies for cancer. *Nat Rev Clin Oncol,* 17: 657–674.

199. Dolmans DE, Fukumura D, Jain RK, 2003, Photodynamic therapy for cancer. *Nat Rev Cancer,* 3: 380–387.

https://doi.org/10.1038/nrc1071

200. Hou X, Tao Y, Pang Y, *et al.*, 2018, Nanoparticle-based photothermal and photodynamic immunotherapy for tumor treatment. *Int J Cancer,* 143: 3050–3060.

https://doi.org/10.1002/ijc.31717

201. Plenagl N, Duse L, Seitz BS, *et al.*, 2019, Photodynamic therapy - hypericin tetraether liposome conjugates and their antitumor and antiangiogenic activity. *Drug Deliv,* 26: 23–33.

https://doi.org/10.1080/10717544.2018.1531954

202. Liu J, Wang L, Shen R, *et al.*, 2022, Novel heptamethine cyanine photosensitizer for fret-amplified photodynamic therapy and two-photon imaging in a-549 cells. *Spectrochim Acta Part A Mol Biomol Spectrosc,* 274: 121083.

https://doi.org/10.1016/j.saa.2022.121083

203. Zhao X, Yang Y, Yu Y, *et al.*, 2019, A cyanine-derivative photosensitizer with enhanced photostability for mitochondria-targeted photodynamic therapy. *Chem Commun (Cambridge, England),* 55: 13542–13545.

https://doi.org/10.1039/C9CC06157F

204. Murren JR, 2002, Modulating multidrug resistance: Can we target this therapy? *Clin Cancer Res,* 8: 633–635.

205. Kessel D, Reiners JJ Jr., 2007, Apoptosis and autophagy after mitochondrial or endoplasmic reticulum photodamage. *Photochem Photobiol,* 83: 1024–1028.

https://doi.org/10.1111/j.1751-1097.2007.00088.x

206. Buytaert E, Dewaele M, Agostinis P, 2007, Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. *Biochim Biophys Acta,* 1776: 86–107.

https://doi.org/10.1016/j.bbcan.2007.07.001

207. Kulbacka J, Choromańska A, Drąg-Zalesińska M, *et al.*, 2020, Proapoptotic activity induced by photodynamic reaction with novel cyanine dyes in caspase-3-deficient human breast adenocarcinoma cell lines (Mcf/Wt and Mcf/Dx). *Photodiagnosis Photodyn Ther,* 30: 101775.

https://doi.org/10.1016/j.pdpdt.2020.101775

208. Krejcir R, Briza T, Sterba M, *et al.*, 2020, Anticancer pentamethinium salt is a potent photosensitizer inducing mitochondrial disintegration and apoptosis upon red light illumination. *J Photochem Photobiol B,* 209: 111939.

https://doi.org/10.1016/j.jphotobiol.2020.111939

209. Krejcir R, Krcova L, Zatloukalova P, *et al.*, 2019, A cyclic pentamethinium salt induces cancer cell cytotoxicity through mitochondrial disintegration and metabolic collapse. *Int J Mol Sci,* 20: 4208.

https://doi.org/10.3390/ijms20174208

210. Wezgowiec J, Kotulska M, Saczko J, *et al.*, 2013, Cyanines in photodynamic reaction assisted by reversible electroporation--*in vitro* study on human breast carcinoma cells. *Photodiagnosis Photodyn Ther,* 10: 490–502.

https://doi.org/10.1016/j.pdpdt.2013.04.004.

211. Flaumenhaft R, Tanaka E, Graham GJ, *et al.*, 2007, Localization and quantification of platelet-rich thrombi in large blood vessels with near-infrared fluorescence imaging. *Circulation,* 115: 84–93.

https://doi.org/10.1161/CIRCULATIONAHA.106.643908

212. Cai Z, Yu J, Hu J, *et al.*, 2023, Three near-Infrared and lysosome-targeting probes for photodynamic therapy (Pdt). *Spectrochim Acta Part A Mol Biomol Spectrosc,* 286: 122027.

https://doi.org/10.1016/j.saa.2022.122027

213. Kong C, Chen X, 2022, Combined photodynamic and photothermal therapy and immunotherapy for cancer treatment: A review. *Int J Nanomed,* 17: 6427–6446.

https://doi.org/10.2147/IJN.S388996

214. Li P, Liu Y, Liu W, *et al.*, 2019, Ir-783 inhibits breast cancer cell proliferation and migration by inducing mitochondrial fission. *Int J Oncol,* 55: 415–424.

https://doi.org/10.3892/ijo.2019.4821

215. Tang Q, Liu W, Zhang Q, *et al.*, 2018, Dynamin-related protein 1-mediated mitochondrial fission contributes to Ir-783-induced apoptosis in human breast cancer cells. *J Cell Mol Med,* 22: 4474–4485.

https://doi.org/10.1111/jcmm.13749

216. Hou L, Yang X, Ren J, *et al.*, 2016, A novel redox-sensitive system based on single-walled carbon nanotubes for chemo-photothermal therapy and magnetic resonance imaging. *Int J Nanomed,* 11: 607–624.

https://doi.org/10.2147/IJN.S98476

217. Wu JB, Shi C, Chu GC, *et al.*, 2015, Near-infrared fluorescence heptamethine carbocyanine dyes mediate imaging and targeted drug delivery for human brain tumor. *Biomaterials,* 67: 1–10.

https://doi.org/10.1016/j.biomaterials.2015.07.028

218. Shao C, Liao CP, Hu P, *et al.*, 2014, Detection of live circulating tumor cells by a class of near-infrared heptamethine carbocyanine dyes in patients with localized and metastatic prostate cancer. *PLoS One,* 9: e88967.

https://doi.org/10.1371/journal.pone.0088967

219. Cohen S, Margel S, 2012, Engineering of near Ir fluorescent albumin nanoparticles for *in vivo* detection of colon cancer. *J Nanobiotechnol,* 10: 36.

https://doi.org/10.1186/1477-3155-10-36

220. Zhang J, Liu Z, Lian P, *et al.*, 2016, Selective imaging and cancer cell death via Ph switchable near-infrared fluorescence and photothermal effects. *Chem Sci,* 7: 5995–6005.

https://doi.org/10.1039/C6SC00221H

221. Thomas RG, Jeong YY, 2017, NIRF heptamethine cyanine dye nanocomplexes for multi modal theranosis of tumors. *Chonnam Med J,* 53: 83–94.

https://doi.org/10.4068/cmj.2017.53.2.83

222. Yang X, Shi C, Tong R, *et al.*, 2010, Near Ir Heptamethine cyanine dye-mediated cancer imaging. *Clin Cancer Res,* 16: 2833–2844.

https://doi.org/10.1158/1078-0432.CCR-10-0059

223. Yuan J, Yi X, Yan F, *et al.*, 2015, Near-infrared fluorescence imaging of prostate cancer using heptamethine carbocyanine dyes. *Mol Med Rep,* 11: 821–828.

https://doi.org/10.3892/mmr.2014.2815

224. Sun C, Wang J, Xia T, *et al.*, 2022, Mitochondrion-targeted NIR therapeutic agent suppresses melanoma by inducing apoptosis and cell cycle arrest via E2f/Cyclin/Cdk pathway. *Pharmaceuticals (Basel, Switzerland),* 15: 1589.

https://doi.org/10.3390/ph15121589

225. Cai H, Wang R, Guo X, *et al.*, 2021, Combining gemcitabine-loaded macrophage-like nanoparticles and erlotinib for pancreatic cancer therapy. *Mol Pharm,* 18: 2495–2506.

https://doi.org/10.1021/acs.molpharmaceut.0c01225

226. Zhan Y, Ma W, Zhang Y, *et al.*, 2019, DNA-based nanomedicine with targeting and enhancement of therapeutic efficacy of breast cancer cells. *ACS Appl Mater Interfaces,* 11: 15354–15365.

https://doi.org/10.1021/acsami.9b03449

227. Hou YJ, Yang XX, Liu RQ, *et al.*, 2020, Pathological mechanism of photodynamic therapy and photothermal therapy based on nanoparticles. *Int J Nanomed,* 15: 6827–6838.

https://doi.org/10.2147/IJN.S269321

228. Zhang Y, Lv T, Zhang H, *et al.*, 2017, Folate and Heptamethine cyanine modified chitosan-based nanotheranostics for tumor targeted near-infrared fluorescence imaging and photodynamic therapy. *Biomacromolecules,* 18: 2146–2160.

https://doi.org/10.1021/acs.biomac.7b00466

229. Rizvi SF, Mu S, Zhao C, *et al.*, 2022, Fabrication of self-assembled peptide nanoparticles for *in vitro* assessment of cell apoptosis pathway and *in vivo* therapeutic efficacy. *Mikrochim Acta,* 189: 53.

https://doi.org/10.1007/s00604-021-05148-7

230. Han X, Wang R, Song X, *et al.*, 2018, A mitochondrial-targeting near-infrared fluorescent probe for bioimaging and evaluating endogenous superoxide anion changes during ischemia/reperfusion injury. *Biomaterials,* 156: 134–146.

https://doi.org/10.1016/j.biomaterials.2017.11.039

231. Cooper E, Choi PJ, Hwang K, *et al.*, 2023, Elucidating the cellular uptake mechanisms of heptamethine cyanine dye analogues for their use as an anticancer drug-carrier molecule for the treatment of glioblastoma. *Chem Biol Drug Des*, 101: 696–716.

https://doi.org/10.1111/cbdd.14171

232. Chi J, Ma Q, Shen Z, *et al.*, 2020, Targeted nanocarriers based on iodinated-cyanine dyes as immunomodulators for synergistic phototherapy. *Nanoscale,* 12: 11008–11025.

https://doi.org/10.1111/cbdd.14171

233. Yu Z, Meng X, Zhang S, *et al.*, 2021, Ir-808 loaded nanoethosomes for aggregation-enhanced synergistic transdermal photodynamic/photothermal treatment of hypertrophic scars. *Biomater Sci,* 10: 158–166.

https://doi.org/10.1039/D1BM01555A

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

K fragment as a polymerase chain reaction-based vector for antibiotic resistance gene hunting

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Abstract

Antimicrobial resistance (AMR) has emerged as an escalating health issue in the global public health arena. To evaluate and predict AMR, it is of utmost importance to identify and characterize both the known and unknown genes responsible for AMR. While known genes can be readily detected, identifying unknown genes present a challenge. In this study, we developed the plasmid K fragment (pKF) by modifying the pUC19 vector, specifically by removing the multiple cloning site and introducing a Prom-RBS sequence. pKF was used for amplification of K fragment that contains a ribosomal binding site (RBS), and promoter at both ends and plasmid origin of replication. The functionality of added Prom-RBS sequence and K fragment as a cloning vector was tested by cloning chloramphenicol resistance gene amplicon and erythromycin resistance gene from genomic DNA, respectively. The cloning experiment demonstrated the usability of this newly developed cloning method with K fragment. K fragment is an innovative vector that can be easily obtained through amplification by polymerase chain reaction and lacks antibiotic resistance markers. This novel approach is convenient to use since it allows cloning of resistance genes at all orientations and this flexibility can be maneuvered by changing restriction enzymes for primers and fragments. With these distinctive features, this vector stands out to be a versatile tool for cloning both known and unknown resistance genes, and the improved method with K fragment enables the microbiological and molecular characterization of cloned genes. K fragment can be utilized for cloning of resistance genes in bacteria originated in different environments without having to perform bacterial culture. We believe that the convenience brought by this technique could lend itself efficient in the battle against the growing AMR crisis through pre-emptive identification of resistance genes.

Keywords: Antimicrobial resistance; Resistance gene; Cloning vector; Cloning method; K fragment

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1. Introduction

The World Health Organization has declared antimicrobial resistance (AMR) as one of the most important health problems in the world and more than 750,000 people die due

to AMR each year[1]. Without showing signs of decreasing, the mortality rate has been projected to reach 10 million in 2050 if necessary and effective steps are not taken to curb AMR[1,2]. The treatment options effective for controlling the infections caused by antimicrobial-resistant pathogens are dwindling due to the emergence of new resistance genes and the spreading of resistant strains[3,4]. One of the drivers promoting the dissemination of AMR is the usage of antibiotics for the treatment of infections in animals and humans, and as a food additive to promote the growth of livestock[5,6]. Due to prevalent usage, the antibiotics remain active and accumulate in the environment[7]. The antibiotic resistance genes appear naturally or due to antibiotic pressure. However, the dissemination of the resistance genes can be accelerated by the antibiotic pressure in the environment, even if the genes occur naturally[8,9]. Therefore, the alarming rise of the AMR scene is a wake-up call for the improvement of new and efficient techniques for the detection of known and unknown antibiotic resistance genes derived from various environments, which is first crucial step for gauging the gravity of the antibiotic resistance problem and formulating solutions to overcome this issue.

In fact, investigations on antibiotic resistance genes are an ongoing effort in the wake of the emergence of antibiotic-resistant strains in clinical isolates and the insufficient treatment of infectious diseases. Phenotypic methods such as antibiogram and microdilution, as well as genotypic methods such as polymerase chain reaction (PCR), are standard methods used in detecting antibiotic resistance in the microbes[10]. However, phenotypic methods are unable to identify the gene or the dominant resistance gene/mechanism contributing to antibiotic resistance, and a culture of microorganisms is needed if phenotypic methods are employed. On the other hand, genotypic methods like PCR cannot be used to detect unknown resistance genes and even the detection of a part of the gene cannot be a corroborative evidence of antibiotic resistance. Cloning of antibiotic resistance genes is a more reliable method, and it has been used to clone the genes from different sources using cloning vectors[11,12]. Functional metagenomics stand as another molecular option for cloning antibiotic resistance genes from different sources[13]. However, the main limitations of this method are as follows: (i) The resistance gene intended to be cloned already exists in the vector and (ii) the high costs and tremendous efforts involved in obtaining the vector needed. In this study, we constructed an extraordinary vector, PCR-based vector named K fragment and conceived a cloning method involving the K fragment. Our results showed that PCR amplicons generated from the amplification using the constructed vector contains plasmid origin of replication sequence that can enable cloning of antibiotic resistance genes.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and antibiotics

Escherichia coli DH10B strain was used as a host organism for cloning experiments. *Staphylococcus aureus* ADU1 and *S. aureus* ADU2 strains having chloramphenicol and erythromycin resistance, respectively, were obtained from our clinical isolates. They were cultured at 37°C in aerobic conditions. Tryptic soy broth and agar were used for cultivation.

Ampicillin, chloramphenicol, and erythromycin were used in this experiment, with final concentrations of 100 µg/ml, 10 µg/ml, and 10 µg/ml, respectively. In addition to ampicillin, 50 µg/ml X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) and 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) were used for the preparation of AXI selective medium.

2.2. Plasmid isolation

Plasmids were isolated using Presto™ Mini Plasmid Kit (Geneaid, China) according to the manufacturer's instructions.

2.3. DNA isolation

DNA was extracted from *S. aureus* ADU1 using DNA4PCR (RTech, Turkey). Briefly, colonies were suspended in distilled water. After centrifugation, the pellet was suspended in DNA4PCR solution. The suspension was incubated at 56°C for 20 min. After vortexing, samples were incubated at 100°C for 8 min. Following centrifugation, the supernatant was used as a DNA source.

Total DNA of *S. aureus* ADU2 was isolated with Bacteria Genomic DNA Purification Kit (Genemarkbio, Taiwan Region). The lysozyme treatment was carried out with a slight modification. Lysostaphin solution with a final concentration of 5 µg/ml was added. The remaining steps were processed according to the manufacturer's recommendations.

2.4. DNA manipulations

Restriction reactions were performed using FastDigest enzymes (Thermo Scientific™, USA). For digestion with one enzyme, 10 µl of DNA, 2 µl of 10× buffer, 1 µl of restriction enzyme, and 6 µl of water were mixed. To perform double digestion, 1 µl of each enzyme and 6 µl of water were pooled together before being added to the reaction mixture. The reaction mixtures were incubated at 37°C for 15 min. For DNA ligation, T4 DNA Ligase

(Thermo Scientific™, USA) was used. Briefly, 10 µl of DNA, 2 µl of 10× buffer, 1 µl of T4 DNA Ligase (5 Weiss U/µl), and 7 µl of water were mixed. The mixtures were incubated at 22°C overnight. S1 Nuclease enzyme (Thermo Scientific™, USA) was used for blunting DNA ends. Briefly, 10 µl of DNA, 6 µl of 5× buffer for S1 Nuclease, 1 µl of S1 Nuclease (1 U/µl), and 13 µl of water were mixed and incubated at room temperature for 30 min. For the phosphorylation of DNA, T4 Polynucleotide Kinase (T4 PNK) was used. Briefly, 10 µl of DNA, 2 µl of 10× buffer, 2 µl of 10 mM ATP, 1 µl of T4 PNK (10 U/µl), and 5 µl of water were mixed and incubated at 37°C for 20 min.

2.5. DNA purification

During all experiments, DNA samples were purified using a PCR clean-up kit (Invitrogen, US).

2.6. Amplification of K fragment

PCR reactions commenced with initial denaturation at 95°C (4 min), followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 30 s to 2 min (depending on the length of amplicon), and final elongation at 72°C for 5 min in a reaction volume of 50 µl. PCR was executed in a T100 thermal cycler (Bio-rad laboratories, Inc, USA). The sequences of primers used in this study are given in Table 1. A total of 4 primers pairs were designed which contain restriction sites for HindIII, EcoRI, PstI, and BamHI. K fragment amplified with BamHI restriction site may also be used for total DNA fragmented with Sau3aI restriction enzyme.

2.7. Sequencing

The cloned DNA fragments were sequenced using primers used for amplification with Sanger sequencing (Medsantek, Turkey).

2.8. Preparation of competent cells and transformation experiments

The competent cells were prepared from *E. coli* DH10B strain, and the transformation process was conducted using the calcium chloride method[14]. Competent cells were also prepared from *S. aureus* RN4220 strain, and the transformation process was executed by means of electroporation[14]. During the electroporation process,

Table 1. Sequences of primers used in this study and their intended purpose, and Prom-RBS sequence

Primer	Sequence (5' → 3')	Intended purpose
P1	TATATATATATTGTCAACAGACCAAGTTTACTCATATATAC	For inserting Prom-RBS
P2	CGGCTAGCATTATATATATATATATTGTCAACAGACC	
P3	AGCTGTACCTCCTTACGGCTAGCATTATATATATATA	
pUCPR	GACAGTTACCAATGCTTAAT	
prmtrSeq	GATCTCAAGAAGATCCTTTG	
promHF	ATGCAAGCTTAGCTGTACCTCCTTACGGC	For functionality testing of inserted Prom-RBS
promER	ATGCGAATTCAGGGCGACACGGAAATGTTG	
cat-HF	ATGCAAGCTTATGACTTTTAATATTATTG	
cat-ER	ATGCGAATTCCTAAATCCAATCATCTAC	
K-F	AGCTGTACCTCCTTACGGC	For testing K fragment as a vector for antibiotic resistance gene cloning
K-R	AGGGCGACACGGAAATGTTG	
K-HF	ATGCAAGCTTAGCTGTACCTCCTTACGGC	
K-HR	ATGCAAGCTTAGGGCGACACGGAAATGTTG	
K-EF	ATGCGAATTCAGCTGTACCTCCTTACGGC	
K-ER	ATGCGAATTCAGGGCGACACGGAAATGTTG	
K-PF	ATGCCTGCAGAGCTGTACCTCCTTACGGC	
K-PR	ATGCCTGCAGAGGGCGACACGGAAATGTTG	
K-BF	ATGCGGATCCAGCTGTACCTCCTTACGGC	
K-BR	ATGCGGATCCAGGGCGACACGGAAATGTTG	
KseqF	CAACATTTCCGTGTCGCCCT	For sequencing the insert K fragment
KseqR	GCCGTAAGGAGGTACAGCT	
Prom-RBS	TTGACAATATATATATATATATATATAATGCTAGCTAAGGAGGTACAGCT	

Notes: *The underlined sequences show restriction sites; H: HindIII; E: EcoRI; P: PstI; and B: BamHI; RBS: Ribosomal binding site.

0.2 cm electroporation cuvette and Ec2 program on MicroPulser Electroporator device (2.49 kV^{-1} pulse) were used (Bio-rad laboratories, Inc, USA).

2.9. Design of Promoter-RBS (Prom-RBS) sequence

E. coli-specific promoter was chosen and obtained from an online resource (http://parts.igem.org)[15] and the selected 17 base sequence contains constitutive and repetitive TA sequence. As Shine-Dalgarno sequence, "TAAGGAGGT" was used[16] and as a spacer, 6 bases, "ACAGCT," were used. The sequences of Prom-RBS and primers used are listed in Table 1.

3. Results

3.1. K fragment

The Prom-RBS sequence and its structure are illustrated in Figure 1A and the schematic representation of K fragment in Figure 1B. The K fragment contains a promoter and RBS at both ends. It was necessary to introduce an additional RBS and promoter sequence, because the fragment with resistance genes may be inserted in any sense which requires RBS and promoter for both orientations for expression of the cloned gene. A plasmid of K fragment (pKF) serving as the template vector for K fragment was constructed. The creation of K fragment entailed two processing steps: (i) Derivation of multiple cloning site (MCS)-free pUC19 ($pUC19^{-MCS}$), and (ii) construction of pKF vector.

3.1.1. Generation of $pUC19^{-MCS}$

MCS was excised from the pUC19 vector using EcoRI and HindIII, MCS was removed from pUC19 using these two enzymes because their restriction sites are found at the ends of MCS and are suitable for the cleaving of the entire MCS region. The cleaved DNA with the 5' overhangs was blunted with S1 Nuclease. The blunt-ended DNA fragments were self-ligated and transferred into *E. coli* DH10B. Contrary to pUC19, MCS-free pUC19 ($pUC19^{-MCS}$) formed white colonies on AXI plate. This was related to the disruption of *lacZalfa* gene due to the removal of MCS. To confirm the MCS removal, the plasmids isolated from transformants were cleaved by RsaI enzyme, ad pUC19 vector was used as a control because one of the three restriction sites of RsaI was found in MCS. Digestion of pUC19 with RsaI generated DNA fragments in three different sizes (241 bp, 676 bp, and 1769 bp). The digestion of $pUC19^{-MCS}$ with RsaI led to the formation of DNA fragments in two different sizes (2010 bp and 676 bp) (Figure 2A). By referring to the patterns of DNA fragments yielded summarized in Figure 2B, we could confirm the removal of MCS from the vector.

3.1.2. The construction of pKF

Prom-RBS sequence designed for this study was introduced with site-directed mutagenesis to the complementary strand and opposite direction of the bla_{TEM} gene in $pUC19^{-MCS}$ to facilitate gene expression at both orientations. Three steps were involved in introducing the sequence due to its length. The workflow of these steps is illustrated in Figure 3A. The PCR products produced during the site-directed mutagenesis process are shown in Figure 3B. Briefly, PCR was performed using P1 and pUCPR as primers and the $pUC19^{-MCS}$ vector as a DNA source. Then, the amplicon was used as a DNA source for the second PCR reaction using P2 and pUCPR primers. The P2 primers contained a part of the promoter and the RBS sequence that we wanted to insert. This second amplicon was used as a DNA source for third PCR reaction which was carried out using P3 primers containing the remaining part of the promoter and RBS sequence (Figure 3B). The final PCR product was phosphorylated and self-ligated. The ligand was transferred to *E. coli* DH10B, and the colonies grown on ampicillin-containing agar were chosen for further investigations. Colony PCR was carried out using prmtrSeq and KsekF

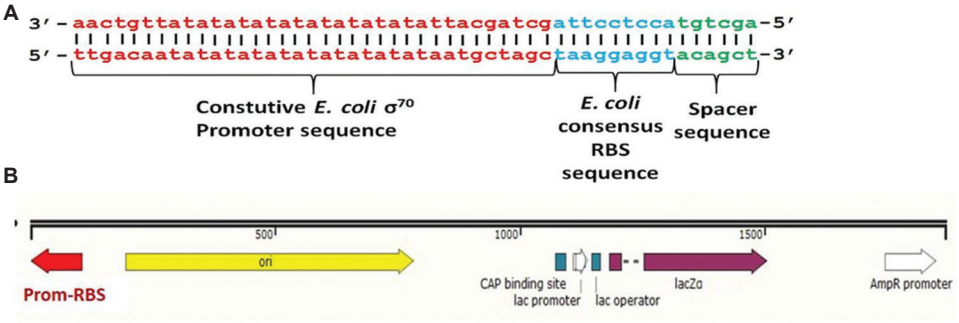

Figure 1. The sequence of Prom-RBS sequence and the schematic representation of K fragment. (A) Prom-RBS sequence includes promoter, ribosomal binding site (RBS) and spacer sequence. (B) K fragment contains promoter and RBS at both terminals. One of them is *ampR* promoter, from pUC19 plasmid. The other promoter was designed in this study. K fragment contains a plasmid origin of replication but has no multiple cloning site region. SnapGene software (from Insightful Science; available at snapgene.com) was used for creating the schematic diagram.

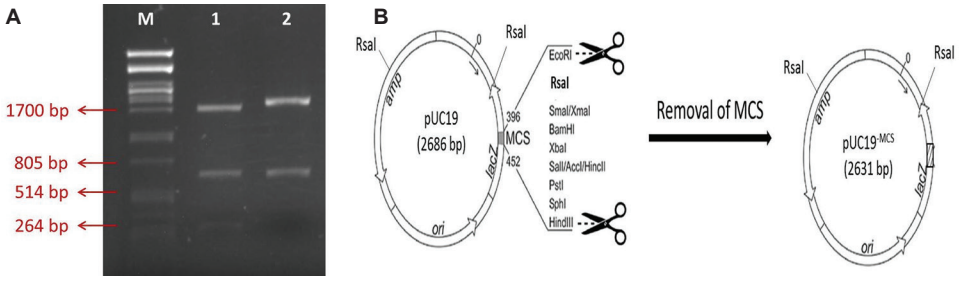

Figure 2. The construction of plasmid K fragment vector. (A) Digestion of MCS-free pUC19 (pUC19^{-MCS}) and pUC19 vectors with RsaI. Lane 1: As a result of the digestion of pUC19 vector with RsaI, three bands were formed, corresponding to fragment sizes of 1769 bp, 676 bp and 241 bp. Lane 2: Following the digestion of pUC19^{-MCS} vector with RsaI, two bands were formed, corresponding to fragment sizes of 2010 bp and 676 bp. M: Lambda-PstI marker. (B) Removal of MCS from pUC19 vector. MCS was excised from pUC19 vector by cleaving with EcoRI and HindIII enzymes and the vector was then self-ligated. The excision of MCS from the vector reduces the number of RsaI restriction sites from 3 to 2 in the vector.

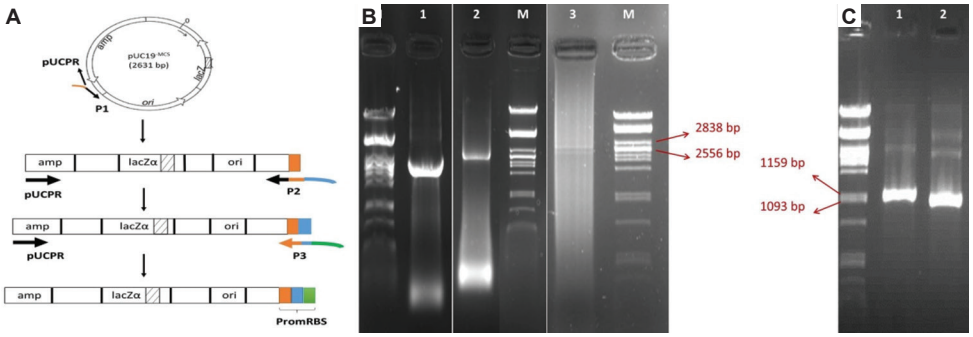

Figure 3. Insertion of Prom-RBS sequence and confirmation with polymerase chain reaction (PCR). (A) The workflow of insertion to pUC19^{-MCS} through the site-directed mutagenesis method. (B) Addition of Prom-RBS into pUC19^{-MCS}. Lane 1: PCR amplicon (2704 bp) obtained using pUC19^{-MCS} as DNA source and P1-pUCPR primer pair. Lane 2: PCR amplicon (2720 bp) obtained using amplicon from Lane 1 as DNA source and P2-pUCPR primer pair. Lane 3: PCR amplicon (2735 bp) obtained using amplicon from Lane 2 as DNA source and P3-pUCPR primer pair. M: Lambda-PstI marker. (C) PCR products amplified from plasmid K fragment (pKF) and pUC19^{-MCS} vectors using prmtrSeq-KsekF primer pair. Lane 1: PCR amplicon was amplified from pKF and its size was 1079 bp. Lane 2: PCR amplicon was amplified from pUC19^{-MCS} and its size was 1031 bp.

primers, and pUC19^{-MCS} was used as a control. The primers used amplify regions that contain Prom-RBS. Thus, if pKF was DNA source, the amplicon size was 1079 bp, but if the Prom-RBS sequence was not inserted into pUC19^{-MCS}, the amplicon size was 1031 bp (Figure 3C). Furthermore, the integration of Prom-RBS into the vector was validated by sequencing. The linear and circular forms of pKF are depicted in Figure 4.

3.2. Confirmation of functionality of Prom-RBS on K fragment

The cloning of *cat* gene was carried out to improve the ability of Prom-RBS sequence on K fragment in facilitating the expression of antibiotic resistance genes. The suitable restriction enzyme sequences were added to both *cat* gene primers and K fragment primers to specify cloning orientation (Figure 5A). K fragment from pKF vector with K-HF and K-ER primers and *cat* from *S. aureus* ADU1 strain with cat-HF and cat-ER primers were amplified with designed primers and the amplicons were cleaved with restriction enzymes (Figure 5B). They were, then, ligated

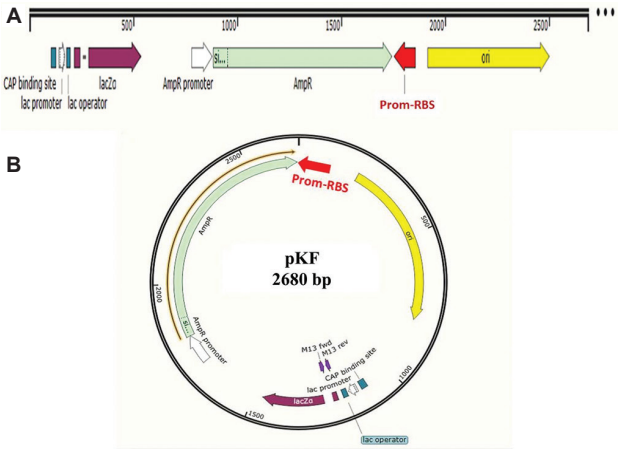

Figure 4. Linear and circular form of plasmid K fragment (pKF) vector. The Prom-RBS sequence that was shown with a red arrow was inserted into the antisense strand of *ampR* to facilitate expression at this strand. (A) The linear form of pKF vector. (B) The circular form of pKF.

and transferred into *E. coli* DH10B cells. The colonies grown on agar plates with chloramphenicol on which the transformed *E. coli* DH10B cells had been plated were

selected. The resistant colonies were subjected to plasmid extraction and colony PCR, through which the presence of K fragment and *cat* gene was confirmed. The plasmid was cleaved with EcoRI and HindIII enzymes, generating K fragment and *cat* gene bands, measuring at 1861 bp and 626 bp in sizes, respectively (Figure 5C). This result showed that the Prom-RBS sequence can facilitate the expression of a resistance gene.

3.3. Usage of K fragment as a vector for antibiotic resistance gene cloning

To test the employability of K fragment for antibiotic resistance gene cloning from genomic DNA, genomic DNA was isolated and subjected to agarose gel electrophoresis (Figure 6A). In addition to chromosome, a band was observed near the level of 2500 bp. K fragment was amplified from pKF vector with designed primers that have HindIII, EcoRI, PstI, and BamHI restriction site sequences at their 5' ends. The primers used are K-HF and K-HR, K-EF and K-ER, K-PF and K-PR, and K-BF and K-BR primer pairs (Table 1). The genomic DNA was digested by HindIII, EcoRI, PstI, and Sau3aI enzymes and K fragment was digested by the same enzymes, except for Sau3aI, for obtaining compatible cohesive ends (Figure 6B). K fragment was digested by BamHI to obtain overhangs at 5' ends of K fragment that are compatible with Sau3aI. The DNA fragments that have the same cohesive ends were ligated and transferred to *E. coli* DH10B. The colonies that grew on erythromycin-containing plate (Figure 7A) were subjected to plasmid isolation and subsequent PCR-based examination. To confirm the existence of K fragment, PCR was performed using K-F and K-R primers. The amplicons were sequenced with KseqF and KseqR primers. The sizes of inserts that give erythromycin resistance cloned in K fragment after the digestion of genomic DNA using HindIII enzyme were equal to each other at about 2400 bp (Figure 7B), whereas the sizes of inserts obtained following the partial digestion of genomic DNA using Sau3aI enzyme

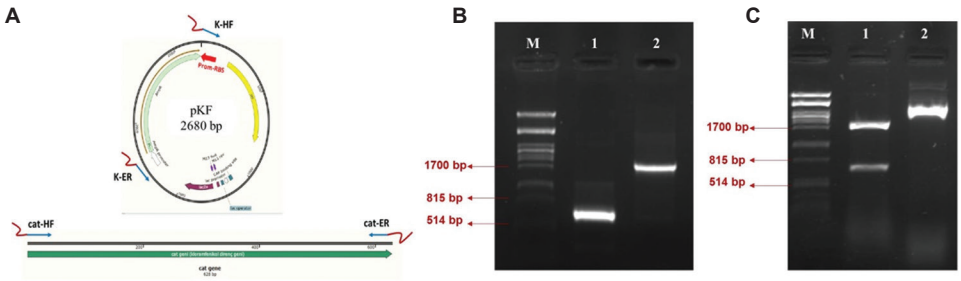

Figure 5. Confirmation of the functionality of Prom-RBS on K fragment by cloning *cat* gene. (A) For the cloning of *cat* to K fragment according to the desired orientation, EcoRI and HindIII restriction site sequences were added to the designed primers used for polymerase chain reaction (PCR). Using primers containing the restriction site sequences, *cat* fragment could be cloned into K fragment in the desired orientation. (B) Confirmation of the presence of K fragment and *cat* fragment in transformants obtained in colonies grown on an agar plate containing chloramphenicol. Lane 1: PCR amplicon obtained from chloramphenicol-resistant transformants using cat-HF and cat-ER primers. Lane 2: PCR amplicon obtained from chloramphenicol-resistant transformants using K-HF and K-ER primers. M line: Lambda-PstI marker. (C) K fragment Ω *cat* plasmid. Lane 1: Two bands representing the fragments of *cat* (626 bp) and K fragment (2861 bp) generated after digestion of the plasmid with EcoRI and HindIII enzymes. Lane 2: Circular form of K fragment Ω *cat* plasmid. M line: Lambda-PstI marker.

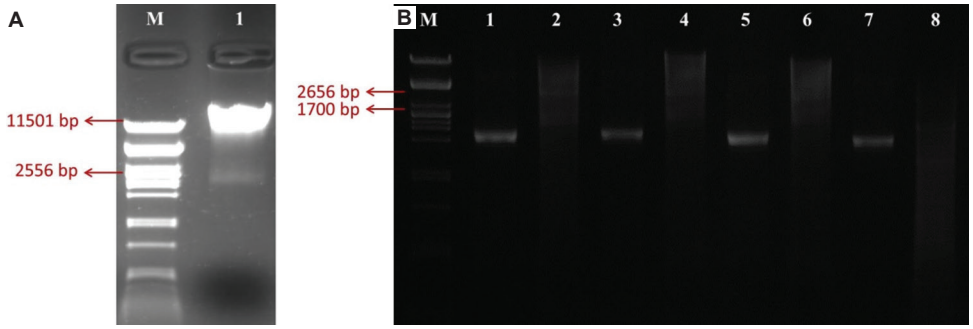

Figure 6. Resistance gene cloning from genomic DNA into K fragment. (A) Genomic DNA of *Staphylococcus aureus* MRSA ADU2 strain. Lane 1: The isolated total DNA and a band that was thought a plasmid with an approximate length of 2500 bp. M line: Lambda-PstI marker. (B) Electrophoresis results of the genomic DNA of MRSA ADU2 strain and K fragment digested by HindIII, EcoRI, PstI, and Sau3AI (BamHI) enzymes. Lanes 1, 3, 5, and 7 represent the electrophoresed products after the digestion of genomic DNA by HindIII, EcoRI, PstI, and Sau3aI, respectively. Lanes 2, 4, 6, and 8 represent the electrophoresed products after the digestion of genomic DNA by HindIII, EcoRI, PstI, and BamHI, respectively.

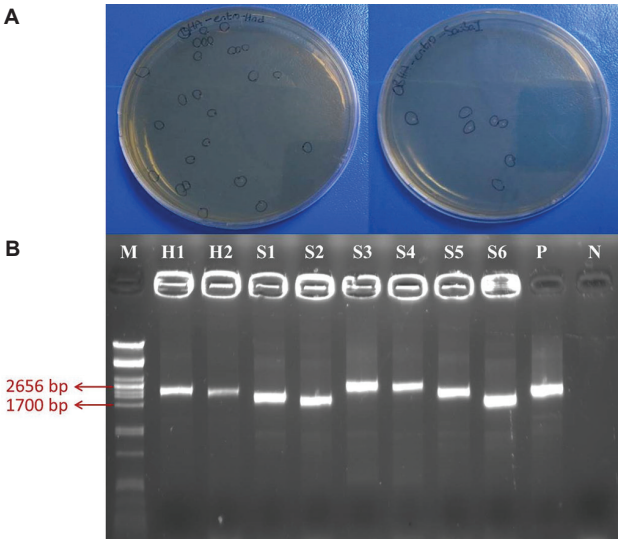

Figure 7. Experiment results of cloning genes from genomic DNA into K fragment. (A) Erythromycin-resistant colonies. Erythromycin resistance gene was cloned into K fragment. The photograph on the left shows the colonies grown on the plate on which the transformants containing the erythromycin-resistant gene cloned into K fragment (with the aid of HindIII enzyme) were plated. The photograph on the right shows the colonies grown on the plate on which teh transformants containing the erythromycin-resistant gene cloned into K fragment (with the aid of Sau3aI [BamHI] enzyme) were plated. (B) The colony PCR results of Erythromycin-resistant colonies. H1 and H2 represent different colonies were obtained by cloning the erythromycin resistance gene into K fragment (with the aid of HindIII enzyme). S1-6 represent different colonies were obtained by cloning the erythromycin resistance gene into K fragment (with the aid of Sau3aI [BamHI] enzyme). P: Positive control, N: Negative control, M: Lambda-PstI marker.

were different, measuring at 2000 bp, 2300 bp, and 2700 bp (Figure 7B). The sequencing results showed that *ermC*, erythromycin resistance gene, was cloned in K fragments in different orientations. The *repL* gene encoding a replication protein in *Staphylococcus* plasmid was cloned to K fragment together with *ermC*. These results revealed that *ermC* gene was carried by a plasmid in *S. aureus* ADU2 strain. The constructed plasmid that can replicate in *E. coli* was transferred to *S. aureus* RN4220 strain by electroporation for confirmation of its ability to replicate in Gram-positive bacteria. After transformation, colonies were selected on erythromycin-containing agar plates and confirmed ability of the construct containing K fragment and *ermC* fragment to replicate in both *E. coli* and *S. aureus*.

4. Discussion

Characterization of antibiotic resistance genes from different sources is the foremost yet important endeavor for deterring the spreading of AMR. Typically, molecular and phenotypic methods are employed in the characterization of resistance genes. In phenotypic methods, however, the bacteria harboring resistance gene need to be cultivable under laboratory conditions, but the challenges lie in the non-cultivability of numerous micro-organisms or the exorbitant costs and immense labor that need to be invested for culturing. Molecular methods are ideal for the detection of known genes but they are incapable of characterizing unknown genes. Thus, cloning and determining resistance genes remain the most feasible approach to characterizing unknown resistance genes. In this study, K fragment was developed to clone antibiotic resistance genes, and its functionality was tested by cloning resistance genes from PCR amplicon and genomic DNA. The present study also demonstrated the capabilities of the newly developed method involving K fragment in phenotypic and molecular characterization of resistance genes.

One of the earliest and most common molecular methods for resistance gene cloning is shotgun cloning[17,18]. Functional metagenomics represent a modified shotgun method designed for cloning antibiotic resistance genes from different environmental sources[19,20]. Despite its usefulness, the shotgun method requires a cloning vector carrying at least one antibiotic resistance gene as a marker and thus cannot be used for cloning all antibiotic resistance genes. K fragment does not carry any antibiotic resistance gene, and it allows cloning and expression of all resistance genes. A conspicuous limitation of using K fragment as a vector is some of the antibiotic resistance cannot be observed in transformed bacteria, rendering the colony identification process challenging after the bacteria are plated on the agar plates. For instance, as the competent bacteria, *E. coli*, are intrinsically resistant to vancomycin, all bacteria will grow on selective media whether or not it contains vancomycin resistance genes in K fragment[21]. The shotgun method also shares the same limitation. We believe that K fragment has a great potential in functional metagenomics for cloning antibiotic resistance gene obtained from various environments (Figure 8). Another popular method for the detection of resistance genes is the total DNA sequencing of a given sample. The metagenomic method is used for the detection of resistance genes in total DNA obtained from different sources[22,23]. However, the resistance genes detected in metagenomic analysis are not inconsistent with the phenotypic and microbiological results[24]. Metagenomic methods are still able to detect DNA sequence of a resistance gene even if it is not complete or functional, but the detectable entities for this method category are only limited to the known resistance genes. Unfortunately, the continuously emerging resistance genes in future will only add to the inexplicable burden that we are facing right now with characterizing the enormous number of unknown resistance genes. Thus, various approaches have been proposed to efficiently identify

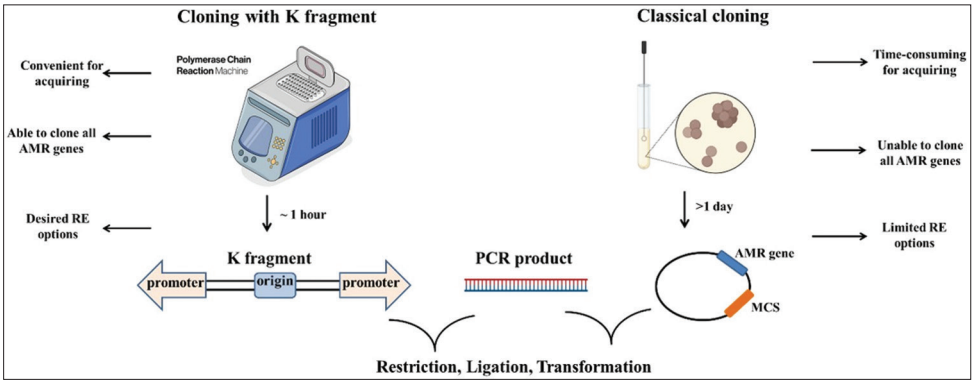

Figure 8. The advantages of the cloning with K fragment and the comparison with classical cloning.
Abbreviations: AMR: Antimicrobial resistance; MCS: Multiple cloning site; PCR: Polymerase chain reaction; RE: Restriction enzyme.

and characterize unknown genes in a high-throughput manner. For instance, one of the bioinformatic approaches involves identifying unknown resistance genes by searching the conserved regions of known resistance genes[25,26]. A different approach entails detecting the antibiotic resistance genes by amplifying and cloning different parts of the integrons[27]. The method is incapable of detecting a large number of resistance genes because many resistance genes are not carried by integrons. Conventional PCR, quantitative PCR (qPCR), and microarray hybridization methods are widely used for the detection of resistance genes in distinct microorganisms or various samples[28]. However, the molecular and metagenomics-based methods are not capable of showing whether detected genes are functional and do not contribute to the characterization of new resistance genes, and most importantly, they are only able to facilitate the detection of known resistance genes. To address the limitations of various approaches in the above, we developed a method in this study that enables the detection of both known and unknown resistance genes and facilitates the characterization of all resistance genes.

5. Conclusion

In this study, we developed K fragment, a PCR-based, antibiotic marker-free vector that allows transcription and translation of resistance genes in all orientations. These attributes lend K fragment useful for the cloning of antibiotic resistance genes. The K fragment can be used for cloning and functional metagenomics of resistance gene obtained from different sources, such as wastewater, feces, and soil. We believe that the vector and new method can facilitate the pre-emptive identification and characterization of antibiotic resistance genes in face of the growing AMR crisis.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

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Writing – original draft: Hanife Salih Doğan, Bülent Bozdoğan
Writing – reviewing & editing: Hanife Salih Doğan, Bülent Bozdoğan

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The sequence of pKF has been submitted to the GenBank database. The accession number is OM304286.

Further disclosure

Part of findings has been presented in the FEMS2023 congress: The 10th FEMS Congress of European Microbiologists. The paper has been uploaded to Research Square preprint server (https://doi.org/10.21203/rs.3.rs-1723923/v1).

References

1. World Health Organization, 2020, Central Asian and European Surveillance of Antimicrobial Resistance: Annual Report 2020 (No. WHO/EURO: 2020-3469-43228-60585). Geneva: World Health Organization. Regional Office for Europe.
2. Dadgostar P, 2019, Antimicrobial resistance: Implications and costs. *Infect Drug Resist*, 12: 3903.

   https://doi.org/10.2147/IDR.S234610
3. Bader MS, Loeb M, Leto D, *et al*., 2020, Treatment of urinary tract infections in the era of antimicrobial resistance and new antimicrobial agents. *Postgrad Med*, 132(3): 234–250.

   https://doi.org/10.1080/00325481.2019.1680052
4. Davies SC, Oxlade C, 2021, Innovate to secure the future: The future of modern medicine. *Future Healthc J*, 8(2): e251–e256.

   https://doi.org/10.7861/fhj.2021-0087
5. Spellberg B, Hansen GR, Kar A, *et al*., 2016, Antibiotic Resistance in Humans and Animals. Indonesia: NAM Perspectives.
6. Patel SJ, Wellington M, Shah RM, *et al*., 2020, Antibiotic stewardship in food-producing animals: Challenges, progress, and opportunities. *Clin Ther*, 42: 1649–1658.

   https://doi.org/10.1016/j.clinthera.2020.07.004
7. Czekalski N, Sigdel R, Birtel J, *et al*., 2015, Does human activity impact the natural antibiotic resistance background? Abundance of antibiotic resistance genes in 21 Swiss lakes. *Environ Int*, 81: 45–55.

   https://doi.org/10.1016/j.envint.2015.04.005
8. Martinez JL, 2009, The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proc Biol Sci*, 276(1667): 2521–2530.

   https://doi.org/10.1098/rspb.2009.0320
9. Bengtsson-Palme J, Kristiansson E, Larsson DGJ, 2018, Environmental factors influencing the development and spread of antibiotic resistance. *FEMS Microbiol Rev*, 42(1): fux053.

   https://doi.org/10.1093/femsre/fux053
10. Fluit AC, Visser MR, Schmitz FJ, 2001, Molecular detection of antimicrobial resistance. *Clin Microbiol Rev*, 14(4): 836–871.

    https://doi.org/10.1128/CMR.14.4.836-871.2001
11. Bozdogan B, Galopin S, Gerbaud G, *et al*., 2003, Chromosomal aadD2 encodes an aminoglycoside nucleotidyltransferase in *Bacillus clausii*. *Antimicrob Agents Chemother*, 47(4): 1343–1346.

    https://doi.org/10.1128/AAC.47.4.1343-1346.2003
12. Brown MG, Mitchell EH, Balkwill DL, 2010, Screening for novel antibiotic resistance genes. *Methods Mol Bio*, 668: 265–271.

    https://doi.org/10.1007/978-1-60761-823-2_18
13. Mullany P, 2014, Functional metagenomics for the investigation of antibiotic resistance. *Virulence*, 5(3): 443–447.

    https://doi.org/10.4161/viru.28196
14. Sambrook J, Russell DW, 2001, Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. p132-150.
15. Wikmark OG, Brautaset T, Agapito-Tenfen SZ, *et al.,* 2016, Synthetic biology-biosafety and contribution to addressing societal challenges.
16. Saito K, Green R, Buskirk AR, 2020, Translational initiation in E. coli occurs at the correct sites genome-wide in the absence of mRNA-rRNA base-pairing. *Elife*, 9: e55002.

    https://doi.org/10.7554/eLife.55002.
17. Thompson CJ, Ward JM, Hopwood DA, 1982, Cloning of antibiotic resistance and nutritional genes in streptomycetes. *J Bacteriol*, 151(2): 668–677.

    https://doi.org/10.1128/jb.151.2.668-677.1982
18. Zhou W, Wang Y, Lin J, 2012, Functional cloning and characterization of antibiotic resistance genes from the chicken gut microbiome. *Appl Environ Microbiol*, 78(8): 3028–3032.

    https://doi.org/10.1128/AEM.06920-11
19. Martiny AC, Martiny JBH, Weihe C, *et al*., 2011, Functional metagenomics reveals previously unrecognized diversity of antibiotic resistance genes in gulls. *Front microbial*, 2: 238.

    https://doi.org/10.3389/fmicb.2011.00238
20. Flórez AB, Vázquez L, Mayo B, 2017, A functional metagenomic analysis of tetracycline resistance in cheese bacteria. *Front Microbiol*, 8: 907.

    https://doi.org/10.3389/fmicb.2017.00907
21. Vestö K, Huseby DL, Snygg I, *et al*., 2018, Muramyl endopeptidase Spr contributes to intrinsic vancomycin resistance in *Salmonella enterica* serovar Typhimurium. *Front Microbiol*, 9: 2941.

    https://doi.org/10.3389/fmicb.2018.02941
22. Fresia P, Antelo V, Salazar C, *et al*., 2019, Urban metagenomics uncover antibiotic resistance reservoirs in coastal beach and sewage waters. *Microbiome*, 7(1): 35.

    https://doi.org/10.1186/s40168-019-0648-z
23. Li Y, Cao W, Liang S, *et al*., 2020, Metagenomic characterization of bacterial community and antibiotic resistance genes in representative ready-to-eat food in southern China. *Sci Rep*, 10(1): 15175.

    https://doi.org/10.1038/s41598-020-72620-4

24. Sanabria-Moreno AM, James Peter JJ, Hjerde E, *et al*., 2021, Shotgun-metagenomics based prediction of antibiotic resistance and virulence determinants in *Staphylococcus aureus* from periprosthetic tissue on blood culture bottles. *Sci Rep*, 11(1): 20848.

https://doi.org/10.1038/s41598-021-00383-7

25. Berglund F, Österlund T, Boulund F, *et al*., 2019, Identification and reconstruction of novel antibiotic resistance genes from metagenomes. *Microbiome*, 7(1): 52.

https://doi.org/10.1186/s40168-019-0670-1

26. Van Camp PJ, Haslam DB, Porollo A, 2020, Prediction of antimicrobial resistance in Gram-negative bacteria from whole-genome sequencing data. *Front Microbiol*, 11: 1013.

https://doi.org/10.3389/fmicb.2020.01013

27. Razavi M, Marathe NP, Gillings MR, *et al*., 2017, Discovery of the fourth mobile sulfonamide resistance gene. *Microbiome*, 5(1): 1–12.

https://doi.org/10.1186/s40168-017-0379-y

28. Galhano BSP, Ferrari RG, Panzenhagen P, *et al*., 2021, Antimicrobial resistance gene detection methods for bacteria in animal-based foods: A brief review of highlights and advantages. *Microorganisms*, 9(5): 923.

https://doi.org/10.3390/microorganisms9050923

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

Continuous topical application of microencapsulated recombinant human epidermal growth factor does not promote the progression of established melanoma in animals

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Abstract

Microencapsulated epidermal growth factor (EGF) device has been applied topically for the management of several types of wounds to accelerate wound healing and prevent scar formation. However, it remains unclear whether such EGF device induced neoplastic transformation in the skin. In this study, we exploited a well-established murine B16-F10 melanoma model, coupled with MTT viability and colony formation assays, to investigate the influence of microencapsulated recombinant EGF (Me-EGF; brand name NewEpi®) and its ingredients on the tumorigenicity of skin cancer cells *in vitro* and *in vivo*. The results indicated that Me-EGF did not stimulate the viability nor the anchorage-dependent growth of B16-F10 melanoma cells. Western blot analysis showed that Me-EGF treatment increased the total and phosphorylated EGFR expression without affecting the HER2 expression in B16-F10 melanoma cells. In mice bearing established B16-F10 melanoma, continuous application of Me-EGF for 14 days did not enhance the melanoma tumor burden compared with control groups. Immunohistochemical analysis also revealed the similar expression of proliferative index Ki-67 between Me-EGF-treated melanoma and other groups. Altogether, these results suggest that the application of Me-EGF device did not promote the oncogenic potential of B16-F10 melanoma *in vitro* and *in vivo*.

Keywords: Melanoma; Epidermal growth factor; Microencapsulation; Me-EGF

1. Introduction

Epidermal growth factor (EGF) is an important mitogen and an excellent wound-healing agent, given its biological functions including regulation of skin cell growth, proliferation, and differentiation[1]. EGF ligand binds to its receptor, EGF receptor (EGFR), and induces EGFR homodimerization or heterodimerization with other member of ErbB family, including ErbB2/HER2, ErbB3/HER3, or ErbB4/HER4, thereby triggering downstream PI3K/AKT or RAS/MEK signaling activation[2]. EGF stimulates re-epithelialization by promoting keratinocyte proliferation and migration to accelerate the healing process of damaged tissues[3]. Despite the numerous benefits in improving chronic wounds and surgical incisions[4,5], the native EGF protein is easily degradable, especially when in liquid form, due to poor biological stability.

Through modification with polysaccharide microencapsulation techniques, the microencapsulated recombinant human EGF (Me-EGF) exhibits improvements in protein stability, integrity, and effectiveness even after long-term storage[6,7]. Accumulating studies have demonstrated that Me-EGF has excellent efficacy to promote healing and reduce scar formation in several types of wounds, including cesarean section and burning. Moreover, Me-EGF also contributes to the rapid repair of osteoarthritis injury[8-10].

The oncogenic potential of EGF remains controversial. Previous studies indicate that high serum levels of EGF were found in patient with melanoma[11]. The elevated EGF levels are associated with melanoma lymph node metastasis; contrarily, EGF knockdown leads to a significant reduction of lymph node metastasis and primary tumor lymphangiogenesis *in vivo*[12]. However, some studies showed that EGF possesses anti-tumor properties. Multiplex cytokines assay reveals that patients with unresectable in-transit melanoma have a lower EGF secretion compared with normal healthy controls[13]. Besides, EGF administration attenuates the growth of xenograft tumors derived from A431 human squamous cell carcinoma cells[14]. Interestingly, the EGF-induced apoptosis is dependent on the expression status of EGFR and HER2[15]. In the present study, we aim to elucidate the influence of microencapsulated recombinant EGF (Me-EGF; brand name NewEpi®) and its ingredients on the tumorigenicity of skin cancer cells using murine B16-F10 melanoma model.

2. Materials and methods

2.1. Cell cultures and reagents

The B16-F10 mouse melanoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 2 mM glutamine, 100 mg/mL streptomycin (Invitrogen; Carlsbad, CA, USA), and 100 U/mL penicillin at 37°C in 5% CO_2 atmosphere. The antibodies used in this work include p-EGFR antibody (sc-101668; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-HER2 antibody (6B12; Cell Signaling Technology, Danvers, MA, USA), EGFR antibody (ab52894; Abcam, Cambridge, MA, USA), Ki-67 antibody (MA5-14520; Thermo Fisher Scientific, Waltham, MA, USA), HER-2 antibody (06-562; Merck KGaA, Darmstadt, Germany), and β-actin antibody (A5441; Sigma-Aldrich, St. Louis, MO, USA). HER2 inhibitor (CP-724714) was purchased from Selleckchem (Houston, TX, USA).

2.2. Preparation of Me-EGF

The human EGF (hEGF) is a small polypeptide containing 53 amino acid residues. NewEpi® (Me-EGF), which contained microencapsulated EGF (10 μg/mL EGF with 0.002% chitosan polysaccharide [Sigma] in excipient [NaCl, 0.9%]), was provided by JoyCom Bio-chem Co. Ltd., Kaohsiung, Taiwan region. Me-EGF was made by JoyCom with a patented technology that microencapsulates EGF with polysaccharide to maintain a complete structure through static electricity stabilized with the negatively charged EGF to form nanoparticles[16,17]. In addition to being able to coat EGF and maintain the integrity of the structure, the polysaccharide has good biocompatibility and biodegradability, can promote wound healing, and is endowed with antibacterial properties.

2.3. Cell proliferation assay

B16-F10 melanoma cells were cultured in a 96-well plate at a density of 1×10^4 cells/mL. After being treated with excipient, polysaccharide, EGF (10 ng/mL), and Me-EGF (10 ng EGF/mL), respectively, for 24 h and 48 h, the cells were supplemented with medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.2 mg/mL) and incubated for 2 h at 37°C. The formazan in viable cells was dissolved with 100 μL of dimethyl sulfoxide (DMSO), and the absorbance was determined using ELISA reader (Dynex Technologies, Inc., Chantilly, VA, USA) at the wavelength of 570 nm.

2.4. Colony formation assay

Cells were cultured in 6-well plates (1×10^3 cells per well) and treated with excipient, polysaccharide, EGF (10 ng/ml), and Me-EGF (10 EGF ng/mL), respectively, once per 2 days for a period of 7 days. At the end of the experiment, the cells were fixed in 4% paraformaldehyde

and incubated in crystal violet (0.01% in 10% buffered formalin; Sigma-Aldrich, St. Louis, MO, USA) for 30 min, followed by washing with tap water and air-drying at room temperature. The number of colonies was photographed and quantified using ImageJ software.

2.5. Melanoma animal model

Four-week-old C57BL/6J male mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan, Republic of China). All animal procedures in this study were approved by Animal Care and Use Committee (IACUC) of National Sun Yat-sen University (approval ID: 11128). To achieve primary melanoma induction, 5×10^5 B16-F10 melanoma cells in 0.1 mL of medium were inoculating into the back of C57BL/6J mice. After implantation for 7 days, tumor mass started to develop in the mice. The tumor-bearing mice were then randomly divided into control, excipient, polysaccharide, and Me-EGF groups (n = 6 per group). Each melanoma in mice was treated daily with topical Me-EGF or other adjuvant (0.1 mL) for 14 days. The tumor volumes were measured with a dial-caliper according to the following formula[18]:

$$\text{Tumor volume } (mm^3) = \text{width}^2 \times \text{length} \times 0.52 \quad \text{(I)}$$

2.6. Histological and immunohistochemical analyses

To examine histological profiles of melanoma tissues treated with Me-EGF and other adjuvants, the paraffin sections were deparaffinized, rehydrated, and stained with modified Mayer's hematoxylin (ab220365; Abcam; Cambridge, MA, USA) and eosin solutions (SI-E6003; Sigma-Aldrich; St. Louis, MO, USA) (H & E). For the analysis of proliferative index in melanoma tissues, the paraffin sections were deparaffinized, blocked with 3% hydrogen peroxide for 10 min, and subjected to antigen retrieval with microwave in 0.01 M citrate buffer for 30 min. The slides were washed three times with phosphate-buffered saline (PBS) and then incubated with Ki-67 antibody (1:500 dilutions in PBS) for 1 h, followed by incubation with secondary antibody for 30 min. The signal was detected using a polymer detection system (Zymed Laboratories, San Francisco, CA). Finally, the slides were counterstained with modified Mayer's hematoxylin, dehydrated, and mounted before microscopic viewing. The percentage of nuclear Ki-67-positive cells was counted from five random images at 200× magnification per melanoma tissue and expressed as mean ± standard deviation (n = 6 each group).

2.7. Western blot analysis

B16-F10 cells were treated with excipient, polysaccharide (0.002%), EGF (100 ng/mL), Me-EGF (100 EGF ng/mL), and CP-724714 (1 μM) in DMEM medium containing 1% FBS for 24 h, respectively. Cell lysates were electrophoresed by means of sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and afterward, the protein bands were transferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked with 5% skim milk in TBS-T for 1 h and then incubated with the indicated primary antibodies (1:1000 dilutions in TBS-T containing 5% FBS) and horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000 dilutions in 5% skim milk) for 1 h, respectively. The signals on the membrane were detected using chemiluminescent HRP substrate (Millipore Corporation; Billerica, MA, USA), and then, the membrane was exposed to X-ray film for autoradiography.

2.8. Statistical analysis

Data were analyzed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA) and expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA), coupled with *post hoc* multiple comparison test using the Tukey procedure, was used to analyze comparisons involving more than two groups. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Administration of Me-EGF did not affect the viability and anchorage-independent growth of B16-F10 melanoma cells *in vitro*

To investigate whether topical application of Me-EGF increased the risk of skin cancers, B16-10 melanoma cells were treated with Me-EGF (10 EGF ng/mL) for 24 h and 48 h, respectively. At the end of experiment, the viability of B16-F10 melanoma cells was determined by MTT assay. The results showed that Me-EGF and its ingredients (excipient, EGF, and polysaccharide) had no significant effect on the proliferation of B16-F10 melanoma cells after 24-h and 48-h incubation (Figure 1). The colony formation assay results showed that Me-EGF had no significant effect on the anchorage-independent growth of B16-F10 cells (Figure 2). Interestingly, polysaccharide exhibited some cytotoxicity to the colony-forming capability of melanoma cells. These findings suggest that the application of Me-EGF did not stimulate the oncogenic behaviors of melanoma cells.

3.2. Me-EGF topical application did not enhance the progression of primary B16-F10 melanoma in C57BL/6J mice

Further, we evaluated the effect of Me-EGF topical application on the tumor growth of melanoma in

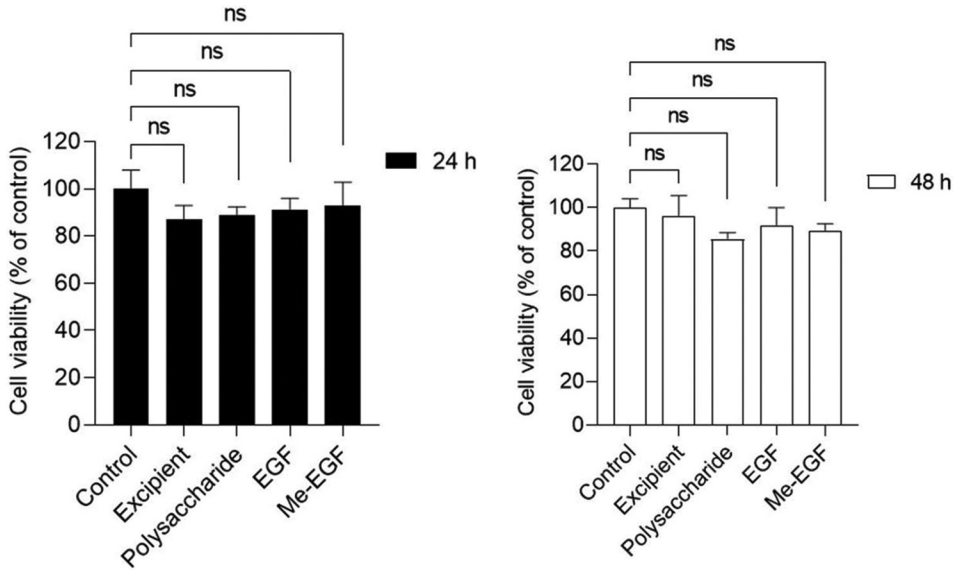

Figure 1. Effect of Me-EGF and its ingredients on the viability of B16-F10 melanoma cells. Cell viability was determined by MTT assay. Data from triplicate experiments are expressed as mean ± SD; data of the untreated control group are expressed as 100%. ns: Not significant.

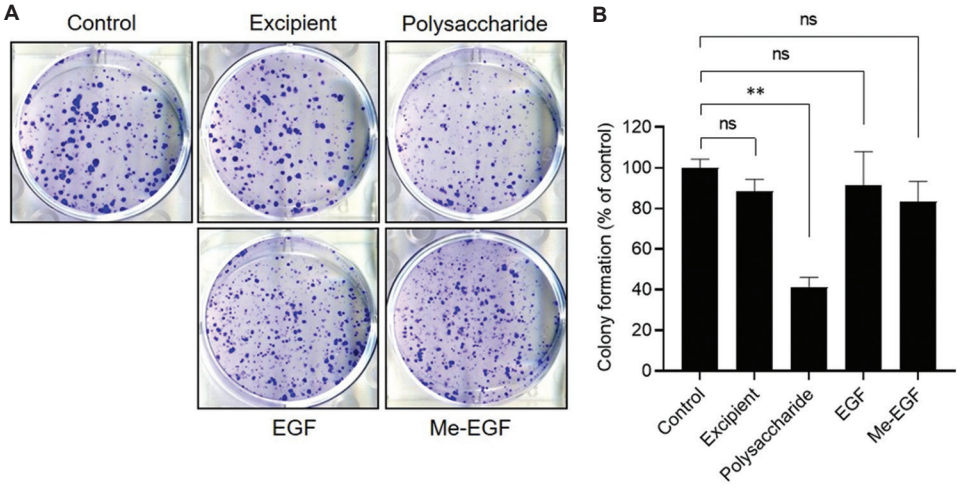

Figure 2. Effect of Me-EGF and its ingredients on colony-forming capability in B16-F10 melanoma cells. (A) Representative images of colonies stained with crystal violet in control and indicated treatment groups. (B) The number of colonies was calculated and expressed as percentage ± SD compared with the results of control group from three different experiments. $^{**}P < 0.01$, ns: Not significant.

established primary melanoma mice model. As shown in Figure 3A, mice were inoculated with B16-F10 melanoma cells (5×10^5 in 0.1 mL of serum medium/per mouse) into the back of mice by subcutaneous injection. After inoculation for 7 days, mice were randomly divided into four groups (control, excipient, polysaccharide, and Me-EGF) and treated once every 2 days for a period of 14 days. Mice were sacrificed and their melanoma tissues were obtained for histological analysis (Figure 3A). The staining results showed that Me-EGF topical application did not promote the tumor growth of primary melanoma; contrarily, a slight growth inhibition relative to the control group was observed, although the difference was not statistically significant (Figure 3B and C). Subsequently, the expression of Ki-67 in Me-EGF and adjuvant-treated melanoma tissues was detected by means of immunohistochemical approach. There was no significant difference in the number of nuclear Ki-67-positive cells in melanoma tissues between control and treated groups (Figure 4). Therefore, these results suggest that Me-EGF topical application does not induce melanoma cell growth in animal model.

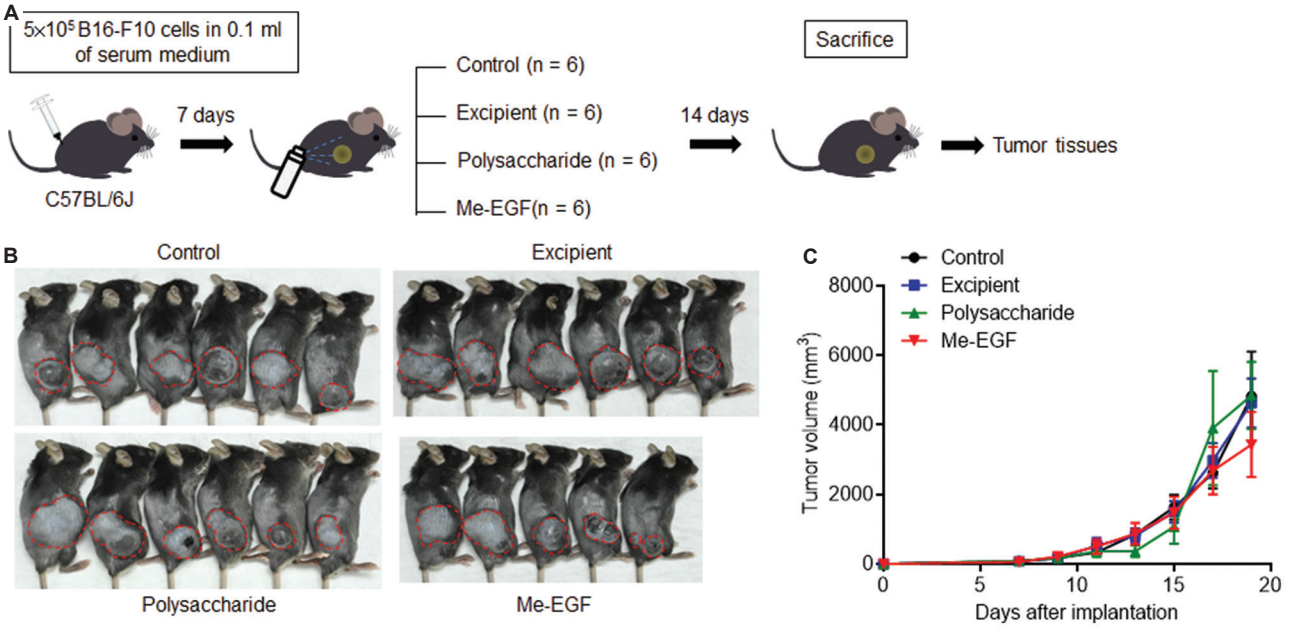

Figure 3. Effect of Me-EGF and its ingredients on the progression of primary melanoma in mice. (A) Schematic diagram depicting the topical administration of Me-EGF in primary melanoma model. (B) Photographs of subcutaneous melanoma tissues (red circle) in mice treated with excipient, polysaccharide, and Me-EGF, respectively. (C) Tumor growth curve of primary melanoma in control and treated groups. Results are expressed as mean ± SD (n = 6/group).

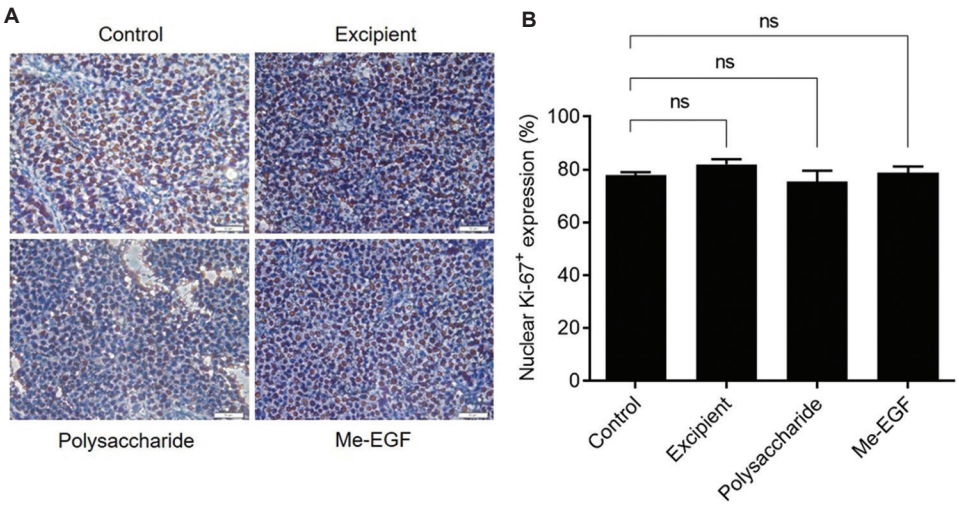

Figure 4. Ki-67 staining of melanoma tissues after treatment with Me-EGF and its ingredients. (A) The representative images of Ki-67 expression in control group, excipient, polysaccharide, and Me-EGF-treated melanoma tissues. Scale bar: 50 μm. (B) Percentage of nuclear Ki-67-positive cells calculated from five random fields (×200 magnification) per melanoma tissue; results are expressed as percentage ± SD (n = 6/group). ns: Not significant.

3.3. Me-EGF increased EGFR expression, but not HER2 expression, in B16-F10 melanoma cells

Given that the expression of ErbB family members is related to the development of cutaneous melanoma,[19] we thus investigated the effect of Me-EGF and its ingredients on the EGFR and HER2 protein levels in B16-F10 melanoma cells. Western blot analysis indicated that the phosphorylated and total EGFR protein levels were increased in EGF- and Me-EGF-treated B16-F10 melanoma cells (Figure 5A). Statistical analysis further revealed that Me-EGF significantly stimulated the expression of both phosphorylated and total EGFR protein compared to control group, although the ratio of p-EGFR/EGFR did not achieve a statistically significant difference (Figure 5B). On the other hand, both EGF and Me-EGF did not impact the HER2 protein levels, regardless of phosphorylated

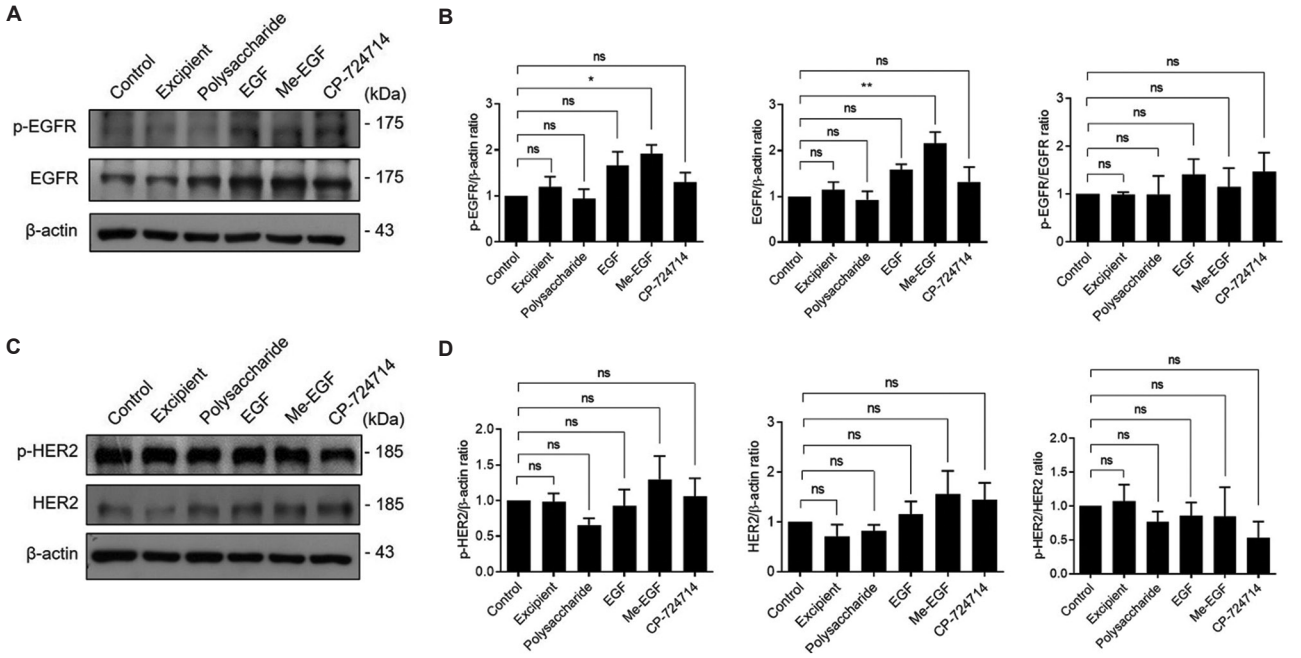

Figure 5. Effect of Me-EGF and its ingredients on the expression of EGFR and HER2 in B16-F10 melanoma cells. (A and C) Western blot analysis of p-EGFR, EGFR, p-HER2, and HER2 expression in B16-F10 melanoma cells treated with adjuvant, Me-EGF, and CP-724714, respectively. The relative protein ratio was normalized with β-actin. (B and D) Bar chart showing the protein ratio; results are expressed as mean ± SD of data from triplicate experiments. **P*<0.05, ***P*<0.01, ns: Not significant.

levels or total protein levels, in B16-F10 melanoma cells (Figure 5C and D). These results suggest that Me-EGF is able to increase EGFR expression, but without affecting HER2 protein level in melanoma cells.

4. Discussion

In this study, we used the patented microencapsulation technology to maintain the integrity of EGF's oligopeptide structure consisting of 53 amino acid. Several human trials have shown that Me-EGF, a preparation of EGF microencapsulated with patented technology, can reduce the formation of scars during cesarean section as well as promote wound healing and osteoarthritis repair[8-10]. To understand the effect of Me-EGF on the growth of cancer cells, we performed experiments to assess the tumorigenicity of melanoma treated with Me-EGF. This study showed that Me-EGF and its ingredients exhibited a slight inhibitory effect on proliferation and colony-forming capability of melanoma cells, whereas chitosan polysaccharide treatment displayed a remarkable inhibitory effect on colony formation. This finding concords with previous studies reporting that chitosan and its derivatives have multiple functions, including antioxidant and antitumor properties[19]. For example, chitosan-encapsulated antioxidant compound can inhibit tube formation of human umbilical vein endothelial cells and glioma growth *in vivo*[20]. Chitosan treatment in human breast cancer cells displays an anti-metastatic activity, which is mediated by the reduction of the activity and amount of matrix metalloproteinase-9 (MMP-9) protein[21].

In addition to *in vitro* cell survival and colony formation tests, we examined the effects of Me-EGF and its ingredients on the growth of melanoma *in vivo*. The primary melanoma model employed in this study had been successfully established and tested in previous studies to evaluate the growth, angiogenesis, and apoptosis of tumor treated with systemic gene therapy, chemotherapy, and small peptide drug administration, further corroborating the reliability and applicability of this model in the assessment of antitumor efficiency[22-24]. The present study showed that both Me-EGF and its ingredients treatment did not significantly influence melanoma growth. Moreover, immunohistochemical analysis indicated that there was no significant difference in the proliferative marker Ki-67 expression in melanoma tissues between control group and treated groups. These results support that topical administration of Me-EGF does not increase the risk of melanoma development.

Numerous studies have reported that the expression profile of ErbB family is associated with several types of cancer, including skin cancer[25-27]. ErbB members, EGFR and HER2, are constitutively expressed in normal

skin, while relatively high percentage of HER2/HER3 co-expression or triple expression of EGFR/HER2/HER3 can be detected in basal cell carcinomas and squamous cell carcinomas compared to normal skin[27]. An ONCOMINE database analysis shows a higher RNA expression of *HER3* in cutaneous melanoma than in normal skin tissues, and under-expression of *EGFR* and *HER2* in cutaneous melanoma, with *EGFR* displaying the lowest expression. There is, however, no significant difference in *HER4* expression between cutaneous melanoma and normal skin tissues[28]. In addition, targeting HER2 by pharmacologic inhibitor is beneficial to the suppression of melanoma cell invasiveness and growth[29,30]. Taken together, these studies indicate that HER2 and HER3 play a more important role in melanoma development. The present study showed that Me-EGF application is able to increase the phosphorylated and total EGFR protein levels in B16-F10 melanoma cells, although the ratio of phosphorylated EGFR to total EGFR protein does not achieve statistically significant difference. Nevertheless, Me-EGF application does not contribute to the activation of phosphorylated and total HER2 proteins in melanoma cells. Thus, these results indicated that the administration of Me-EGF might not trigger HER2-mediated oncogenic signaling pathway. On the other hand, some clinical studies have shown that treating patients with recombinant EGF protein did not increase the incidence of cancer[31,32]. Further studies should be conducted to investigate whether other ErbB family members, especially HER3, are activated in Me-EGF-treated melanoma cells.

We acknowledge several limitations in this study. First, the topical application of Me-EGF, which is prepared as liquid-based formulation, posed some challenges in terms of absorption and penetration, affecting drug adhesion to the skin surface of mice. Second, the B16-10 melanoma mouse model may not fully recapitulate the clinical and genetic features of human melanoma. Future experiments are warranted to overcome the above-mentioned shortcomings.

5. Conclusion

Topical Me-EGF application does not promote melanoma tumor growth *in vitro* and *in vivo*.

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Conflict of interest

The authors have declared no conflicts of interest.

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All co-authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures in this study were approved by Animal Care and Use Committee (IACUC) of National Sun Yat-sen University (approval ID: 11128).

Consent for publication

Not applicable.

Availability of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Yao Q, Yu Z, Liu P, *et al.*, 2019, High efficient expression and purification of human epidermal growth factor in *Arachis Hypogaea* L. *Int J Mol Sci*, 20(8): 2045.

   https://doi.org/10.3390/ijms20082045

2. Gurtner GC, Werner S, Barrandon Y, *et al.*, 2008, Wound repair and regeneration. *Nature*, 453(7193): 314–321.

   https://doi.org/10.1038/nature07039

3. Werner S, Grose R, 2003, Regulation of wound healing by growth factors and cytokines. *Physiol Rev*, 83(3): 835–870.

   https://doi.org/10.1152/physrev.2003.83.3.835

4. Tiaka EK, Papanas N, Manolakis AC, *et al.*, 2012, Epidermal growth factor in the treatment of diabetic foot ulcers: An update. *Perspect Vasc Surg Endovasc Ther*, 24(1): 37–44.

   https://doi.org/10.1177/1531003512442093

5. Hardwicke J, Schmaljohann D, Boyce D, *et al.*, 2008, Epidermal growth factor therapy and wound healing--past, present and future perspectives. *Surgeon*, 6(3): 172–177.

   https://doi.org/10.1016/s1479-666x(08)80114-x

6. Mao S, Sun W, Kissel T, 2010, Chitosan-based formulations

for delivery of DNA and siRNA. *Adv Drug Deliv Rev*, 62(1): 12–27.

https://doi.org/10.1016/j.addr.2009.08.004

7. Kean T, Thanou M, 2010, Biodegradation, biodistribution and toxicity of chitosan. *Adv Drug Deliv Rev*, 62(1): 3–11.

https://doi.org/10.1016/j.addr.2009.09.004

8. Kao CC, Huang SY, Chiang CH, *et al*., 2021, Microencapsulated rhEGF to facilitate epithelial healing and prevent scar formation of cesarean wound: A randomized controlled trial. *Taiwan J Obstet Gynecol*, 60(3): 468–473.

https://doi.org/10.1016/j.tjog.2021.03.014

9. Tsai HC, Sheng C, Chang LS, *et al*., 2021, Chitosan-microencapsulated rhEGF in promoting wound healing. *J Wound Care*, 30(Sup9a): IXi–IXxi.

https://doi.org/10.12968/jowc.2021.30.Sup9a.IX

10. Lin SC, Zhang X, Chen SY, *et al*., 2021, Microencapsulated recombinant human epidermal growth factor ameliorates osteoarthritis in a murine model. *Evid Based Complement Alternat Med*, 2021: 9163279.

https://doi.org/10.1155/2021/9163279

11. Treskova I, Topolcan O, Windrichova J, *et al*., 2018, OPG, OPN, EGF and VEGF levels at individual breslow score stages in malignant melanoma. *Anticancer Res*, 38(8): 4907–4911.

https://doi.org/10.21873/anticanres.12806

12. Bracher A, Cardona AS, Tauber S, *et al*., 2013, Epidermal growth factor facilitates melanoma lymph node metastasis by influencing tumor lymphangiogenesis. *J Invest Dermatol*, 133(1): 230–238.

https://doi.org/10.1038/jid.2012.272

13. Shetty G, Beasley GM, Sparks S, *et al*., 2013, Plasma cytokine analysis in patients with advanced extremity melanoma undergoing isolated limb infusion. *Ann Surg Oncol*, 20(4): 1128–1135.

https://doi.org/10.1245/s10434-012-2785-5

14. Lim YJ, Jeon SR, Koh JM, *et al*., 2015, Tumor growth suppression and enhanced radioresponse by an exogenous epidermal growth factor in mouse xenograft models with A431 cells. *Cancer Res Treat*, 47(4): 921–930.

https://doi.org/10.4143/crt.2014.153

15. Choi J, Moon SY, Hong JP, *et al*., 2010, Epidermal growth factor induces cell death in the absence of overexpressed epidermal growth factor receptor and ErbB2 in various human cancer cell lines. *Cancer Invest*, 28(5): 505–514.

https://doi.org/10.3109/07357900902783179

16. Lee DW, Lim C, Israelachvili JN, *et al*., 2013, Strong adhesion and cohesion of chitosan in aqueous solutions. *Langmuir*, 29(46): 14222–14229.

https://doi.org/10.1021/la403124u

17. Andrade F, Goycoolea F, Chiappetta DA, *et al*., 2011, Chitosan-grafted copolymers and chitosan-ligand conjugates as matrices for pulmonary drug delivery. *Int J Carbohydr Chem*, 2011: 865704.

https://doi.org/10.1155/2011/865704

18. Liu GS, Liu LF, Lin CJ, *et al*., 2006, Gene transfer of pro-opiomelanocortin prohormone suppressed the growth and metastasis of melanoma: Involvement of alpha-melanocyte-stimulating hormone-mediated inhibition of the nuclear factor kappaB/cyclooxygenase-2 pathway. *Mol Pharmacol*, 69(2): 440–451.

https://doi.org/10.1124/mol.105.015404

19. Wang W, Xue C, Mao X, 2020, Chitosan: Structural modification, biological activity and application. *Int J Biol Macromol*, 164: 4532–4546.

https://doi.org/10.1016/j.ijbiomac.2020.09.042

20. Kim S, Gaber MW, Zawaski JA, *et al*., 2009, The inhibition of glioma growth *in vitro* and *in vivo* by a chitosan/ellagic acid composite biomaterial. *Biomaterials*, 30(27): 4743–4751.

https://doi.org/10.1016/j.biomaterials.2009.05.010

21. Nam KS, Shon YH, 2009, Suppression of metastasis of human breast cancer cells by chitosan oligosaccharides. *J Microbiol Biotechnol*, 19(6): 629–633.

https://doi.org/10.4014/jmb.0811.603

22. Liu GS, Tsai HE, Weng WT, *et al*., 2011, Systemic pro-opiomelanocortin expression induces melanogenic differentiation and inhibits tumor angiogenesis in established mouse melanoma. *Hum Gene Ther*, 22(3): 325–335.

https://doi.org/10.1089/hum.2010.090

23. Wu JC, Tsai HE, Hsiao YH, *et al*., 2020, Topical MTII therapy suppresses melanoma through PTEN upregulation and cyclooxygenase II inhibition. *Int J Mol Sci*, 21(2): 681.

https://doi.org/10.3390/ijms21020681

24. Weng CH, Wu CS, Wu JC, *et al*., 2020, Cisplatin-induced giant cells formation is involved in chemoresistance of melanoma cells. *Int J Mol Sci*, 21(21): 7892.

https://doi.org/10.3390/ijms21217892

25. Majumder A, Sandhu M, Banerji D, *et al*., 2021, The role of HER2 and HER3 in HER2-amplified cancers beyond breast cancers. *Sci Rep*, 11(1): 9091.

https://doi.org/10.1038/s41598-021-88683-w

26. Watanabe S, Yonesaka K, Tanizaki J, *et al*., 2019, Targeting of the HER2/HER3 signaling axis overcomes ligand-mediated resistance to trastuzumab in HER2-positive breast cancer. *Cancer Med*, 8(3): 1258–1268.

https://doi.org/10.1002/cam4.1995

27. Krahn G, Leiter U, Kaskel P, *et al*., 2001, Coexpression

patterns of EGFR, HER2, HER3 and HER4 in non-melanoma skin cancer. *Eur J Cancer*, 37(2): 251–259.

https://doi.org/10.1016/s0959-8049(00)00364-6

28. Liu S, Geng R, Lin E, *et al*., 2021, ERBB1/2/3 expression, prognosis, and immune infiltration in cutaneous melanoma. *Front Genet*, 12: 602160.

https://doi.org/10.3389/fgene.2021.602160

29. Ma J, Han H, Liu D, *et al*., 2013, HER2 as a promising target for cytotoxicity T cells in human melanoma therapy. *PLoS One*, 8(8): e73261.

https://doi.org/10.1371/journal.pone.0073261

30. Pudelko K, Wieland A, Hennecke M, *et al*., 2022, Increased microtubule growth triggered by microvesicle-mediated paracrine signaling is required for melanoma cancer cell invasion. *Cancer Res Commun*, 2(5): 366–379.

https://doi.org/10.1158/2767-9764.CRC-22-0010

31. Wu HG, Song SY, Kim YS, *et al*., 2009, Therapeutic effect of recombinant human epidermal growth factor (RhEGF) on mucositis in patients undergoing radiotherapy, with or without chemotherapy, for head and neck cancer: A double-blind placebo-controlled prospective phase 2 multi-institutional clinical trial. *Cancer*, 115(16): 3699–3708.

https://doi.org/10.1002/cncr.24414

32. Wang GY, Xia ZF, Zhu SH, *et al*., 2003, Clinical observation of the long-term effects of rhEGF on deep partial-thickness burn wounds. *Zhonghua Shao Shang Za Zhi*, 19(3): 167–168.

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

Exploring the "Carpenter" as a substrate for green synthesis: Biosynthesis and antimicrobial potential

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Abstract

The frequent use of antibiotics has created favorable conditions for bacteria to develop resistance. Bacterial resistance is a global health issue, causing at least 1 million deaths worldwide annually. The quest for new and effective antimicrobials with activities against resistant bacteria demands immediate attention. The use of nanoparticles as alternatives to conventional antibiotics may have the potential to combat bacterial resistance. Silver nanoparticles, in particular, have captivated the interest of most researchers by virtue of their broad-range antimicrobial activity against bacteria, stemming from their strong biocidal effect on microorganisms. Conventionally, silver nanoparticles have been synthesized through physical, chemical, and biological processes. However, the biosynthesis of silver nanoparticles from the wings of carpenter bees (*Xylocopa virginica*), abundantly available in summer in the United States of America, is yet to be explored. In this study, we report the synthesis of silver nanoparticles using wing extracts from *X. virginica*. Subsequently, the biosynthesized nanoparticles were characterized using ultraviolet-visible (UV-Vis) absorption spectroscopy and scanning electron microscopy (SEM). Furthermore, we investigated the antimicrobial activity of the biosynthesized nanoparticles against two common Gram-negative and Gram-positive pathogenic bacteria, namely, *Klebsiella pneumonia, Escherichia coli, Micrococcus luteus*, and *Staphylococcus aureus*, using microdilution method. The study outcomes indicate that biosynthesized silver nanoparticles from *X. virginica* wing extract demonstrated an absorption band at 440 nm, and SEM revealed spherical nanoparticles with sizes ranging from 20–60 nm. In addition, biosynthesized silver nanoparticles from the wings of *X. virginica* exhibited antimicrobial activity against all the tested bacteria, signifying their potential in biomedical, pharmaceutical, and agricultural applications.

Keywords: Carpenter bee; *Xylocopa virginica*; Nanoparticles; Antimicrobial; Bacteria; Green synthesis

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1. Introduction

The fortuitous discovery of antibiotics about a century ago led to the identification of a wide range of antimicrobials, revolutionizing the control of infectious diseases, and significantly improving health and life expectancy[1-3]. However, the inappropriate use of these antibiotics in recent years has become a key factor contributing to antibiotic resistance, leading to the emergence of "superbugs"[4-6]. Antimicrobial resistance poses a global threat, resulting in the death of at least 1.2 million people every year[7]. In the United States of America, more than 2 million cases of antimicrobial-resistant infections occur annually, associated with at least 20,000 deaths[8,9]. Consequently, there is a pressing need for alternative strategies to address the growing issues associated with antibiotic resistance.

Nanotechnology has captured the attention of scientists around the world as a potential solution to overcome bacterial resistance patterns[10-13]. Metallic nanoparticles have demonstrated antimicrobial properties against bacteria through mechanisms such as oxidative stress, cations release, or non-oxidative processes[14]. Many scientists have used various techniques, including chemical, physical, and green synthesis methods, to synthesize nanoscale metals with specific shapes and dimensions[15-17].

Green synthesis, also known as "eco-friendly nano-factories," holds greater value than traditional chemical and physical synthesis. In comparison to chemical and physical methods, green synthesis is more affordable and sustainable[18-20]. Green synthesis utilizes various organisms, such as bacteria, yeast, fungi, algal species, plants, and even the wings of certain insects, to serve as substrates for nanomaterial synthesis[21-24]. Despite the versatility of green synthesis, there are no existing reports on the synthesis of nanoparticles from the wings of *Xylocopa virginica*. Recognizable by its brightly colored body and/or wings (Figure 1)[25], *X. virginica*, commonly known as the carpenter bee, is a group of bees that, as the name suggests, tunnel and nest in wood using their strong jaws to create galleries for rearing their young. After 7 weeks, newly formed adults leave the tunnels and embark on foraging for food. The lifespan of adult males is typically shorter, often around 1 year, with many dying shortly after mating. In contrast, adult females can live for more than 2 years.

While researchers have explored the antimicrobial properties of insect wings[26-29], the wings of *X. virginica* have received relatively less attention. Therefore, the present study seeks to expand the scope of nanotechnological applications in insects by utilizing deceased adult wings of *X. virginica* for the synthesis of silver nanoparticles. The synthesized silver nanoparticles were thoroughly evaluated for their antimicrobial activities (against bacterial pathogens) and antioxidant properties. This study introduces an alternative to chemically produced nanoparticles, presenting new avenues for scientists engaged in the battle against antimicrobial resistance.

Figure 1. Adult *Xylocopa virginica*.
Adopted from: https://www.environmentalpestcontrol.ca/bees/carpenter-bee.

2. Materials and methods

2.1. *X. virginica* wings collection and preparation

The wings of deceased *X. virginica* used in the present study were collected in June 2022 from the Winston Salem State University campus in North Carolina, USA, and transported to the laboratory. In the laboratory, the wings were gently incised with the aid of forceps, followed by washing twice with distilled water to remove particulate matter. Subsequently, the wings were stored in sterile Petri dishes until needed.

2.2. Biosynthesis and characterization of silver nanoparticles

The green synthesis of silver nanoparticles from *X. virginica* was carried out following previously described methodologies[30-32]. Briefly, 0.1 g of *X. virginica* wings was weighed and added to 10 ml of distilled water, followed by incubation at 90°C for 60 min. Subsequently, the sample was centrifuged at 8000 rpm for 10 min. The supernatant (*X. virginica* wing extract) was collected, and its pH was adjusted to neutral. Next, 1 ml of the *X. virginica wing* extract was added to 49 mL of 1 mM silver nitrate ($AgNO_3$) solution at 28 ± 1°C for 60 min to synthesize silver nanoparticles. The formation of nanoparticles was visually inspected, and the absorbance spectrum of the reaction was measured using GENESYS™ 180 UV-Vis Spectrophotometer (Fisher Scientific, USA) within the range of 200–1000 nm. The spectrophotometer operated

at a resolution of 1 nm at room temperature. Furthermore, the biosynthesized nanoparticles were characterized using a scanning electron microscope (SEM) (JEOL JSM-IT800 HL, JEOL Ltd, Japan) at the Joint School of Nanoscience and Nanoengineering, University of North Carolina at Greensboro and North Carolina A and T State University, Greensboro, North Carolina, USA.

2.3. Antimicrobial activity

The antimicrobial activity of biologically synthesized silver nanoparticles was evaluated using the broth microdilution method[29,33], a simple method employed to determine the minimum inhibitory concentration. The antibacterial activity of the biosynthesized nanoparticles was evaluated against common pathogenic strains of both Gram-positive (*Staphylococcus aureus* [ATCC 25923] and *Micrococcus luteus* [ATCC 4698]) and Gram-negative (*Escherichia coli* 1946 [ATCC 25922] and *Klebsiella pneumoniae* NCTC 9633 [ATCC 13883]) bacteria. Bacterial cultures were treated with different concentrations of the biosynthesized silver nanoparticles (ranging from 0 – 100 µM) and subsequently incubated at 37°C with agitation at 120 rpm in a shaking incubator. The experiment was performed in triplicates to ensure reliability. Bacterial growth was assessed at 5 and 24 h using a 98-well plate format Glomaxmulti plate reader (Promega, USA). In addition, to further observe the antimicrobial activity of the synthesized silver nanoparticles, untreated and treated bacterial samples were collected for SEM analysis following the method described by Tian *et al.*[34]. Briefly, bacterial samples were centrifuged at 8000 rpm for 10 min and washed three times with phosphate-buffered saline (PBS). Subsequently, the samples were fixed in 2.5% glutaraldehyde solution (configured with PBS) overnight at 4°C, followed by incubation in increasing concentration of ethanol. The samples were immediately pre-frozen at −20°C, freeze-dried for 12 h, and observed using SEM.

2.4. Statistical analysis

The data obtained were expressed as means ± standard deviation using GraphPad Prism 8.0. Statistical comparisons between groups were conducted using the Student's *t*-test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Synthesis of silver nanoparticles

X. virginica wings extract reduced silver nitrate into silver nanoparticles. The change of color from yellow to dark brown (Figure 2) after a 60-min incubation period was strongly indicated the successful formation of silver nanoparticles. This phenomenon is attributed to the excitation of the surface plasmon resonance (SPR) effect, a characteristic process in the generation of such nanoparticles. In addition, confirmation of the biosynthesis of silver nanoparticles was obtained through UV-vis spectroscopy. The spectroscopic analysis revealed a maximum peak absorbance at 440 nm (Figure 3), confirming the presence of the synthesized nanoparticles.

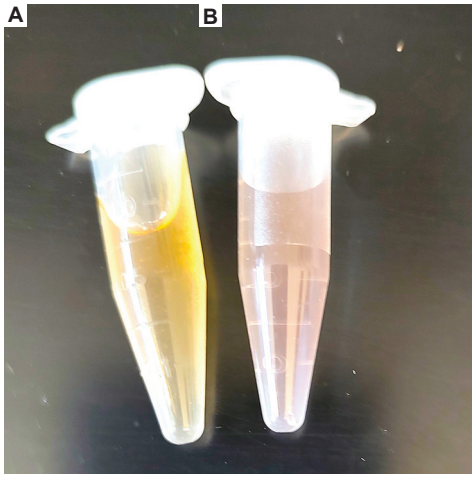

Figure 2. Color variations before and after the reduction of silver nitrate by *Xylocopa virginica* wings extract into silver nanoparticles. (A) *X. virginica* wings extract with no silver nitrate. (B) *X. virginica* wings extract with added silver nitrate.

3.2. SEM analysis of biosynthesized nanoparticles from *X. virginica* wings extract

The SEM analysis revealed the size ranges and morphology of the nanoparticles synthesized from *X. virginica* wings. The results indicated the presence of spherical-shaped nanoparticles with a size range between 10 and 40 nm (Figure 4).

3.3. Antimicrobial activity of *X. virginica*-derived silver nanoparticles

Antibacterial activity of biosynthesized silver nanoparticles derived from *X. virginica* was evaluated against *K. pneumonia*, *E coli*, *M. luteus*, and *S. aureus* at two time points (i.e., 5 and 24 h). As indicated by the results obtained through the broth microdilution method, the growth of all tested bacteria exhibited a reduction with an increase in nanoparticle concentration (Figures 5–8). The highest concentration (100 µM) of the synthesized nanoparticles significantly inhibited the growth of all tested bacteria compared to the control at both 5 and 24 h ($P < 0.05$). Notably, the graphical representations underscored that Gram-negative strains, *K. pneumonia* and *E. coli*, were most sensitive to the biosynthesized silver nanoparticles, exhibiting substantial growth inhibition at both 5 and 24 h.

All bacterial samples, including both treated and control groups, were thin sectioned for SEM imaging

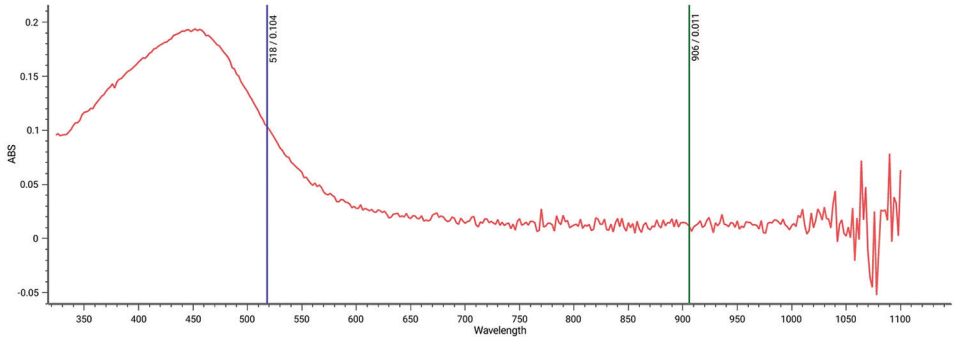

Figure 3. Ultraviolet–visible spectroscopy depicting the absorption spectrum of 290 µM silver nanoparticles biosynthesized from *Xylocopa virginica* wings extract.

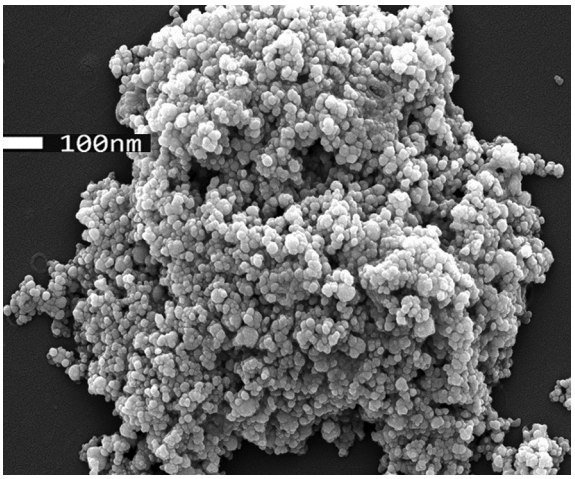

Figure 4. Representative scanning electron microscopy micrograph of biosynthesized synthesized silver nanoparticles derived from *Xylocopa virginica.*

to illuminate the mechanism of the antimicrobial interaction. Specifically, samples treated with 100 µM of the synthesized nanoparticles and incubated for 24 h were selected. This selection was based on the observed manifestation of their most potent antimicrobial effects among the synthesized nanoparticles at different concentrations, strongly implying a significant interaction between the nanoparticles and the surface components of bacterial cells. As observed in SEM micrographs, the biosynthesized nanoparticles exhibited aggregation and interaction on the surface of *K. pneumonia*, *E coli*, *M. luteus*, and *S. aureus* (Figure 9B, 9D, 9F, and 9H). In contrast, these aggregations and interactions were absent in the control groups (Figure 9A, 9C, 9E, and 9G).

4. Discussion

This study aims to demonstrate a cost-effective and sustainable approach for the synthesis of silver nanoparticles involving the utilization of carpenter bees, *X. virginica*, and to explore their potential antimicrobial activity against *K. pneumonia, E. coli, M. luteus*, and *S. aureus*. The distinct color change in the aqueous silver nitrate solution on interaction with *X. virginica* wing extract within 60 min confirmed the reduction of silver ions to silver nanoparticles, resulting in surface plasmon vibration[35-37]. The phenolic compounds present in the wings were hypothesized to act as reducing and stabilizing agents in the synthesis of silver nanoparticles, a proposition further supported by the well-defined absorption band at 440 nm. The strongest absorption peak observed at 440 nm correlates with surface plasmon resonance, a characteristic feature of silver nanoparticles[38]. The previous studies have demonstrated that silver nanoparticles exhibit UV-vis absorption maximum in the range of 400–500 nm due to surface plasmon vibration, as observed in various sources such as Geranium leaf extract (*Strychnos potatorum*), wild mushroom (*Ganoderma sessiliforme*), termites (*Mang mao*), and American roaches (*Periplaneta americana*)[32,38-40].

The size range and morphology of the nanoparticles synthesized from *X. virginica* were further confirmed through SEM micrographs. The SEM micrographs depict the regular, spherical shape of individual silver nanoparticles as well as several aggregates, characterized by smooth edges with sizes ranging from 20–40 nm. Similar findings were reported by Jakinala *et al.*[32], Kagithoju *et al.*[38], and Jain *et al.*[41] in studies involving the synthesis of nanoparticles using extracts from *M. mao*, *Carica papaya*, and *Pseudomonas canadensis*, respectively. The efficacy of nanoparticles is enhanced by tailoring their physical characteristics, such as shape[42,43]. Spherical silver nanoparticles, in particular, exhibited the most potent antimicrobial activity against *E. coli*. This superiority is attributed to their highest surface area and smallest size, allowing for a higher release rate of silver ions and consequently improving antimicrobial activity[44].

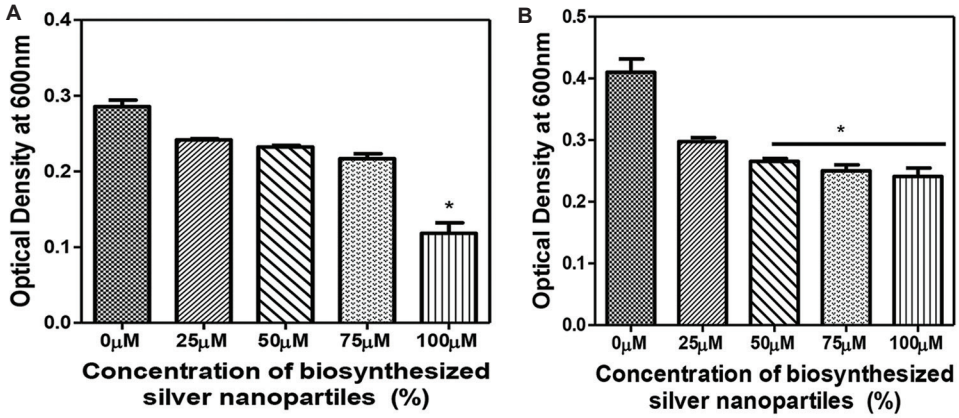

Figure 5. Antibacterial activities of *Xylocopa virginica*-derived silver nanoparticles against *Klebsiella pneumonia*. (A) 5 h after exposure. (B) 24 h after exposure. **P* < 0.05.

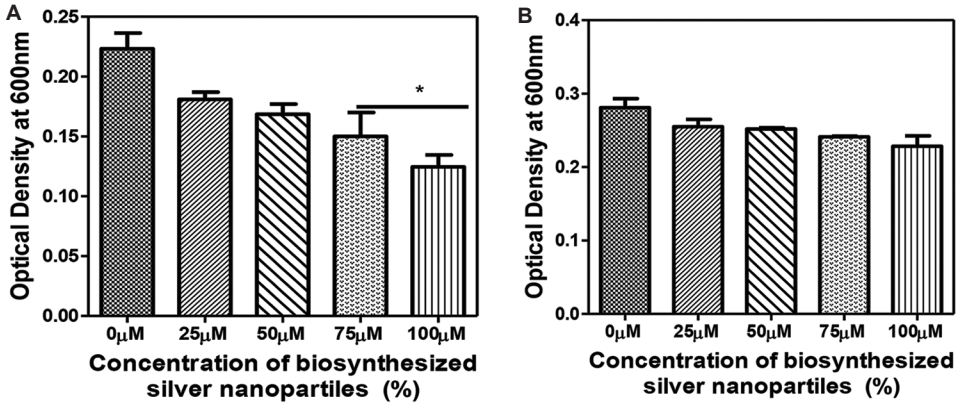

Figure 6. Antibacterial activities of *Xylocopa virginica*-derived silver nanoparticles against *Escherichia coli*. (A) 5 h after exposure. (B) 24 h after exposure. **P* < 0.05.

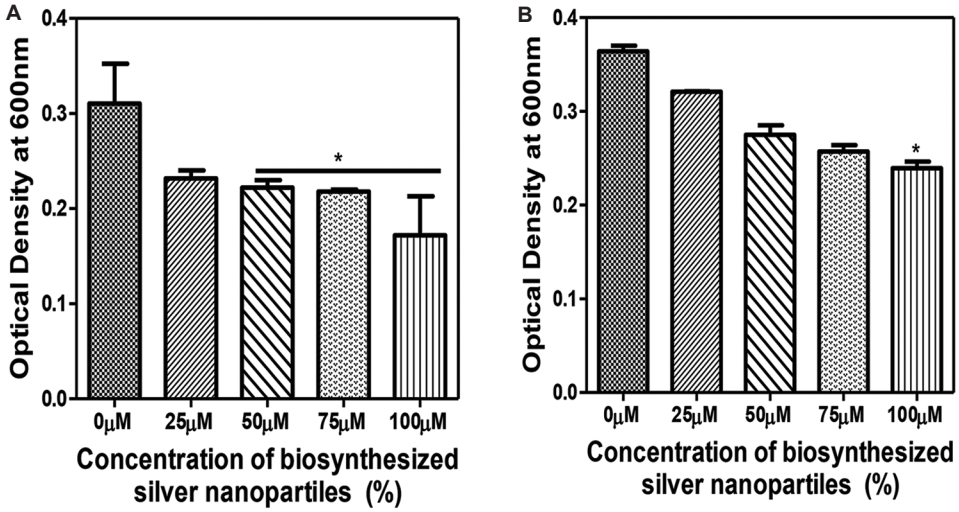

Figure 7. Antibacterial activities of *Xylocopa virginica*-derived silver nanoparticles against *Micrococcus luteus*. (A) 5 h after exposure. (B) 24 h after exposure. **P* < 0.05.

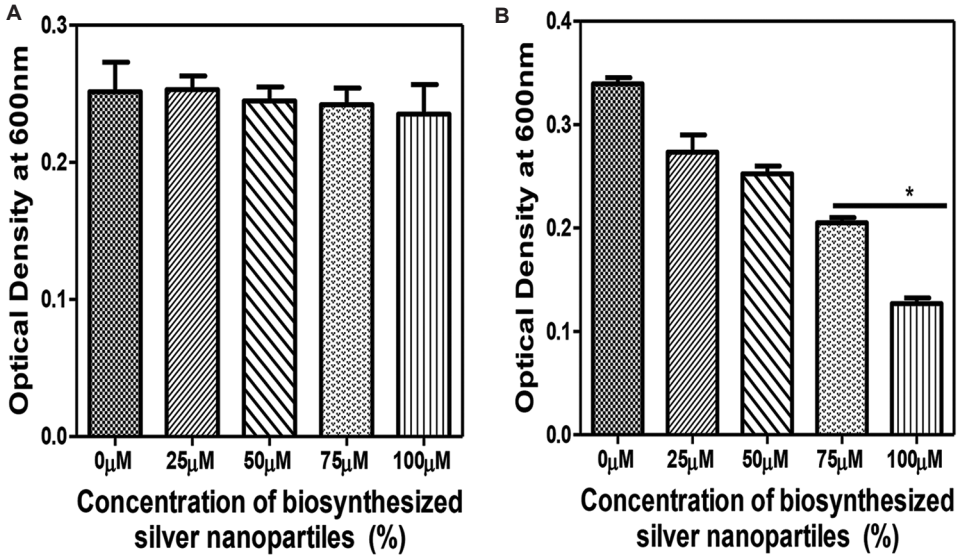

Figure 8. Antibacterial activities of *Xylocopa virginica*-derived silver nanoparticles against *Staphylococcus aureus.* (A) 5 h after exposure. (B) 24 h after exposure. *$P < 0.05$.

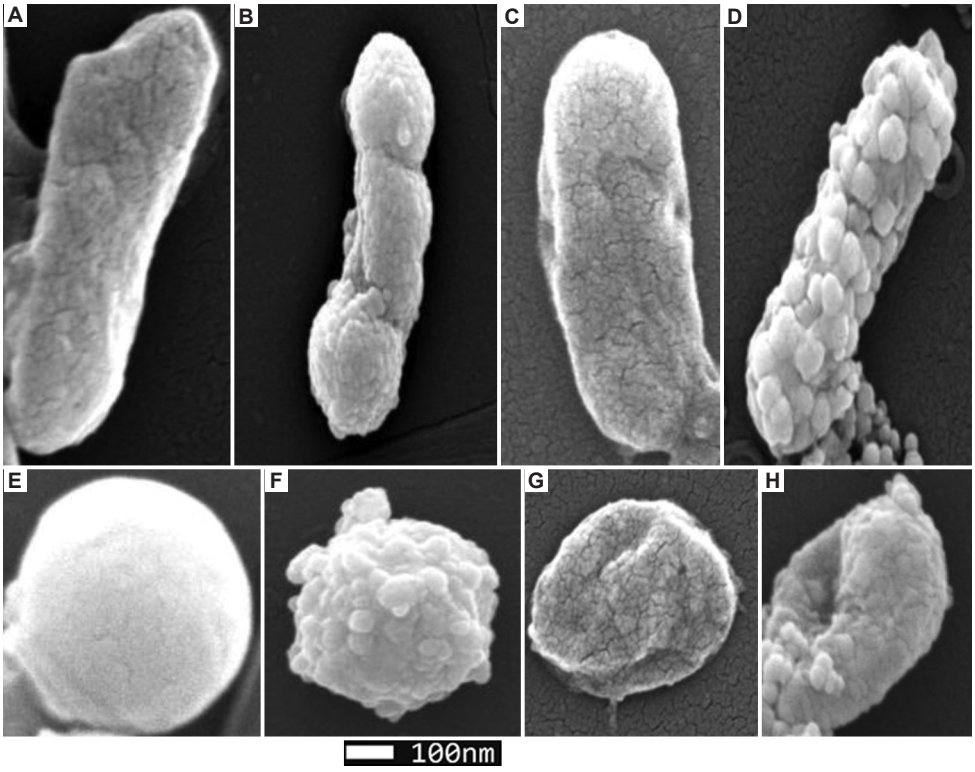

Figure 9. Scanning electron microscopy micrographs of bacterial cells in treated and control groups after incubation with *Xylocopa virginica*-derived biosynthesized nanoparticles at 24 h. (A) Control *Klebsiella pneumonia* cell; (B) *K. pneumonia* cell with nanoparticles; (C) control, *Escherichia coli*; (D) *E. coli* with nanoparticles; (E) control *Staphylococcus aureus*; (F) control *S. aureus* with nanoparticles; (G) control *Micrococcus luteus*; and (H) *M. luteus* with nanoparticles. Scale bar: 100 nm.

Biosynthesized silver nanoparticles from *X. virginica* exhibited potential antimicrobial activity at both 5 and 24 h post-treatment. The growth of *K. pneumonia*, *E. coli*, *M. luteus*, and *S. aureus* decreased with an increase in nanoparticle concentration. SEM micrographs (Figure 9) demonstrated that the silver nanoparticles adhered to

the surfaces, revealing their antimicrobial activity against these tested microbes. The interaction between silver nanoparticles and microbes typically occurs through electrostatic attraction and the affinity of silver ions to sulfur- and/or phosphorus-containing compounds in bacterial cells[45,46]. Our data indicate that Gram-negative bacteria were more sensitive to biosynthesized silver nanoparticles, corroborating the findings in other studies. This heightened sensitivity in Gram-negative bacteria is attributed to their thick lipopolysaccharide layer and thin peptidoglycan layer in the cell wall[47,46]. In contrast, Gram-positive bacterial cells, characterized by thin lipopolysaccharide layers and thick peptidoglycan layers in their cell wall, demonstrated reduced sensitivity. This finding suggests that the thick peptidoglycan layers may limit the uptake of silver nanoparticles[47,48].

Silver nanoparticles exhibit high effectiveness against a broad spectrum of microbes. However, the exact mechanisms underlying their antimicrobial potential are still under investigation[49]. Recent studies suggest that on binding to bacterial surfaces, silver nanoparticles can readily release silver ions, thereby increasing the permeability of the cytoplasmic membrane and disrupting cellular components[50-52]. The disruption of bacterial cell walls and membranes can result in morphological changes in bacterial cells[53,54]. The advantageous combination of a high surface area and small size, particularly in the case of spherical nanoparticles, facilitates a more rapid release of silver ions, consequently enhancing antimicrobial effects[44]. Carpenter bees are known for excavating holes into woods to create galleries for their eggs and larvae. Our data demonstrate that the "carpenter" is also a substrate for green synthesis, which shows potential antimicrobial activity.

5. Conclusion

In this study, we explored an eco-friendly and rapid, green approach for the synthesis of silver nanoparticles using the wings of carpenter bees, *X. virginica* wings. The silver nanoparticles biosynthesized from carpenter bees demonstrated effectiveness against *K. pneumonia*, *E. coli*, *M. luteus*, and *S. aureus*, suggesting the potential application of this approach in the production of nanomedicines.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Akamu J. Ewunkem
Formal analysis: Akamu J. Ewunkem
Investigation: Akamu J. Ewunkem, Zahirah J. Williams, Niore S. Johnson
Methodology: All authors
Writing – original draft: Akamu J. Ewunkem,
Writing – review & editing: Akamu J. Ewunkem, Zahirah J. Williams, Justice L. Brittany

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The data that support the findings of this study are available on request from the corresponding author.

References

1. Aminov RI, 2010, A brief history of the antibiotic era: Lessons learned and challenges for the future. *Front Microbiol*, 1: 134.

   https://doi.org/10.3389/fmicb.2010.00134

2. Khan S, Hussain A, Attar F, *et al.*, 2022, A review of the berberine natural polysaccharide nanostructures as potential anticancer and antibacterial agents. *Biomed Pharmacother*, 146: 112531.

   https://doi.org/10.1016/j.biopha.2021.112531

3. Murugaiyan J, Kumar PA, Rao GS, *et al.*, 2022, Progress in alternative strategies to combat antimicrobial resistance: Focus on antibiotics. *Antibiotics* (*Basel*), 11(2): 200.

   https://doi.org/10.3390/antibiotics11020200

4. Mahmoudi H, 2020, Bacterial co-infections and antibiotic resistance in patients with COVID-19. *GMS Hyg Infect Control*, 15: Doc35.

   https://doi.org/10.3205/dgkh000370

5. Nwobodo DC, Ugwu MC, Anie CO, *et al.*, 2022, Antibiotic resistance: The challenges and some emerging strategies for tackling a global menace. *J Clin Lab Anal*, 36: e24655.

https://doi.org/10.1002/jcla.24655

6. Daria S, Islam MR, 2022, Indiscriminate use of antibiotics for COVID-19 treatment in South Asian countries is a threat for future pandemics due to antibiotic resistance. *Clin Pathol*, 15.

https://doi.org/10.1177/2632010X221099889

7. Band VI, Weiss DS, 2019, Heteroresistance: A cause of unexplained antibiotic treatment failure? *PLoS Pathogens*, 15(6): e1007726.

https://doi.org/10.1371/journal.ppat.1007726

8. Flynn CE, Guarner J, 2023, Emerging antimicrobial resistance. *Mod Pathol*, 36: 100249.

https://doi.org/10.1016/j.modpat.2023.100249

9. Stewart H, 2023, Alexander Fleming, antibiotic resistance, and relevant lessons for the mitigation of risk from advanced artificial intelligence.

10. Baptista PV, McCusker MP, Carvalho A, *et al*., 2018, Nano-strategies to fight multidrug resistant bacteria-'A battle of the titans'. *Front Microbiol*, 9: 1441.

https://doi.org/10.3389/fmicb.2018.01441.

11. Kumar R, Aadil KR, Ranjan S, *et al*., 2020, Advances in nanotechnology and nanomaterials-based strategies for neural tissue engineering. *J Drug Deliv Sci Technol*, 57: 101617.

https://doi.org/10.1016/j.jddst.2020.101617

12. Munir MU, Ahmed A, Usman M, *et al*., 2020, Recent advances in nanotechnology-aided materials in combating microbial resistance and functioning as antibiotics substitutes. *Int J Nanomedicine*, 15: 7329–7358.

https://doi.org/10.2147/IJN.S265934

13. Pasika SR, Bulusu R, Rao BV, *et al*., 2023, Nanotechnology for biomedical applications. In: Nanomaterials: Advances and Applications. Singapore: Springer Nature Singapore. p297–327.

14. Wang L, Hu C, Shao L, 2017, The antimicrobial activity of nanoparticles: Present situation and prospects for the future. *Int J Nanomed*, 12: 1227–1249.

https://doi.org/10.2147/IJN.S121956

15. Razavi M, Salahinejad E, Fahmy M, *et al*., 2015, Green chemical and biological synthesis of nanoparticles and their biomedical applications. In: Green Processes for Nanotechnology: From Inorganic to Bioinspired Nanomaterials. Germany: Springer. p207–235.

16. Nadaroglu H, Güngör AA, İnce S. 2017, Synthesis of nanoparticles by green synthesis method. *Int J Innov Res Rev*, 1(1): 6–9.

17. Aswathi VP, Meera S, Maria CA, *et al*, 2023, Green synthesis of nanoparticles from biodegradable waste extracts and their applications: A critical review. *Nanotechnol Environ Eng*, 8(2): 377–97.

18. Singh J, Dutta T, Kim KH, *et al*., 2018, Green'synthesis of metals and their oxide nanoparticles: Applications for environmental remediation. *J Nanobiotechnology*, 16(1): 84.

https://doi.org/10.1186/s12951-018-0408-4

19. Alsammarraie FK, Wang W, Zhou P, *et al*., 2018, Green synthesis of silver nanoparticles using turmeric extracts and investigation of their antibacterial activities. Colloids Surf B Biointerfaces, 171: 398–405.

https://doi.org/10.1016/j.colsurfb.2018.07.059

20. Devi HS, Boda MA, Shah MA, *et al*., 2019, Green synthesis of iron oxide nanoparticles using *Platanus orientalis* leaf extract for antifungal activity. *Green Process Synth*, 8(1): 38–45.

https://doi.org/10.1515/gps-2017-0145

21. Lahiri D, Nag M, Sheikh HI, *et al*., 2021, Microbiologically synthesized nanoparticles and their role in silencing the biofilm signaling cascade. *Front Microbiol*, 12: 636588.

https://doi.org/10.3389/fmicb.2021.636588

22. Elsakhawy T, Omara AE, Abowaly M, *et al*., 2022, Green synthesis of nanoparticles by mushrooms: A crucial dimension for sustainable soil management. *Sustainability*, 14(7): 4328.

https://doi.org/10.3390/su14074328

23. Selvakesavan RK, Franklin G, 2021, Prospective application of nanoparticles green synthesized using medicinal plant extracts as novel nanomedicines. *Nanotechnol Sci Appl*, 14: 179–195.

https://doi.org/10.2147/NSA.S333467

24. Ajaykumar AP, Sabira O, Sebastian M, *et al*., 2023, A novel approach for the biosynthesis of silver nanoparticles using the defensive gland extracts of the beetle, *Luprops tristis* Fabricius. *Sci Rep*, 13(1): 10186.

25. Stavenga DG, 2023, Pigmentary colouration of hairy carpenter bees, genus *Xylocopa*. *Naturwissenschaften*, 110(3): 22.

https://doi.org/10.1007/s00114-023-01854-9

26. Ivanova EP, Hasan J, Webb HK, *et al*., 2012, Natural bactericidal surfaces: Mechanical rupture of *Pseudomonas aeruginosa* cells by cicada wings. *Small*, 8(16): 2489.

https://doi.org/10.1002/smll.201200528

27. Jakinala P, Lingampally N, Hameeda B, *et al*., 2020, Insect wing extract: A novel source for green synthesis of nanoparticles of antioxidant and antimicrobial potential. *bioRxiv*, 2020-10.

https://doi.org/10.1101/2020.10.21.348458

28. Soni A, Brightwell G, 2022, Nature-inspired antimicrobial surfaces and their potential applications in food industries. *Foods*, 11(6): 844.

https://doi.org/10.3390/foods11060844

29. Ewunkem AJ, A'lyiha FB, Justice BL, *et al*., 2023, Honeybee wings hold antibiofouling and antimicrobial clues for improved applications in health care and industries. *AIMS Microbiol*, 9(2): 332.

https://doi.org/10.3934/microbiol.2023018

30. Tszydel M, Sztajnowski S, Michalak M, *et al*., 2009, Structure and physical and chemical properties of fibres from the fifth larval instar of caddis-flies of the species *Hydropsyche angustipennis*. *Fibres Text East Eur*, 6(77): 7–12.

31. Lateef A, Ojo SA, Azeez MA, *et al*., 2016, Cobweb as novel biomaterial for the green and eco-friendly synthesis of silver nanoparticles. *Appl Nanosci*, 6: 863–874.

https://doi.org/10.1007/s13204-015-0492-9

32. Jakinala P, Lingampally N, Hameeda B, *et al*., 2021, Silver nanoparticles from insect wing extract: Biosynthesis and evaluation for antioxidant and antimicrobial potential. *PLoS One*, 16(3): e0241729.

https://doi.org/10.1371/journal.pone.0241729

33. Eloff JN, 1998, A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Med*, 64: 711–713.

https://doi.org/10.1055/s-2006-957563

34. Tian B, Liu Y, Chen D, 2021. Adhesion behavior of silica nanoparticles with bacteria: Spectroscopy measurements based on kinetics, and molecular docking. *J Mole Liquids*, 343: 117651.

https://doi.org/10.1016/j.molliq.2021.117651

35. Veerasamy R, Xin TZ, Gunasagaran S, *et al*., 2011, Biosynthesis of silver nanoparticles using mangosteen leaf extract and evaluation of their antimicrobial activities. *J Saudi Chem Soc*, 15(2): 113–120.

https://doi.org/10.1016/j.jscs.2010.06.004

36. Mohanta YK, Nayak D, Biswas K, *et al*., 2018, Silver nanoparticles synthesized using wild mushroom show potential antimicrobial activities against food borne pathogens. *Molecules*, 23(3): 655.

https://doi.org/10.3390/molecules23030655

37. Farrag HM, Mostafa FA, Mohamed ME, 2020, Green biosynthesis of silver nanoparticles by *Aspergillus niger* and its antiamoebic effect against *Allovahlkampfia spelaea* trophozoite and cyst. *Exp Parasitol*, 219: 108031.

https://doi.org/10.1016/j.exppara.2020.108031

38. Kagithoju S, Godishala V, Nanna RS, 2015, Eco-friendly and green synthesis of silver nanoparticles using leaf extract of *Strychnos potatorum* Linn. F. and their bactericidal activities. *3 Biotech*, 5: 709–714.

39. Pileni MP, 2000, Fabrication and physical properties of self-organized silver nanocrystals. *Pure Appl Chem*, 72(1–2): 53–65.

https://doi.org/10.1351/pac200072010053

40. Khatami M, Iravani S, Varma RS, *et al*., 2019, Cockroach wings-promoted safe and greener synthesis of silver nanoparticles and their insecticidal activity. *Bioprocess Biosyst Eng*, 42: 2007–2014.

41. Jain D, Kachhwaha S, Jain R, *et al*., 2010, Novel microbial route to synthesize silver nanoparticles using spore crystal mixture of *Bacillus thuringiensis*. *Indian J Exp Biol*, 48: 1152–1156.

42. Lim YH, Tiemann KM, Hunstad DA, *et al*., 2016, Polymeric nanoparticles in development for treatment of pulmonary infectious diseases. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 8(6): 842–871.

https://doi.org/10.1002/wnan.1401

43. Makabenta JM, Nabawy A, Li CH, *et al*., 2021, Nanomaterial-based therapeutics for antibiotic-resistant bacterial infections. *Nat Rev Microbiol*, 19(1): 23–36.

https://doi.org/10.1038/s41579-020-0420-1

44. Sayed FA, Eissa NG, Shen Y, *et al*., 2022, Morphologic design of nanostructures for enhanced antimicrobial activity. *J Nanobiotechnol*, 20(1): 1–18.

https://doi.org/10.2147/IJN.S246764

45. Yin IX, Zhang J, Zhao IS, *et al*., 2020, The antibacterial mechanism of silver nanoparticles and its application in dentistry. *Int J Nanomed*, 2020: 2555–2562.

https://doi.org/10.2147/IJN.S246764

46. More PR, Pandit S, Filippis AD, *et al*., 2023, Silver nanoparticles: Bactericidal and mechanistic approach against drug resistant pathogens. *Microorganisms*, 11(2): 369.

https://doi.org/10.3390/microorganisms11020369

47. Meikle TG, Dyett BP, Strachan JB, *et al*., 2020, Preparation, characterization, and antimicrobial activity of cubosome encapsulated metal nanocrystals. *ACS Appl Mater Interfaces*, 12(6): 6944–6954.

https://doi.org/10.1021/acsami.9b21783

48. Pazos-Ortiz E, Roque-Ruiz JH, Hinojos-Márquez EA, *et al*., 2017, Dose-dependent antimicrobial activity of silver nanoparticles on polycaprolactone fibers against gram-positive and gram-negative bacteria. *J Nanomater*, 2017: 4752314.

https://doi.org/10.1155/2017/4752314

49. Malarkodi C, Rajeshkumar S, Paulkumar K, *et al*., 2013, Biosynthesis of semiconductor nanoparticles by using sulfur reducing bacteria *Serratia nematodiphila*. *Adv Nano Res*, 1: 83.

https://doi.org/10.12989/anr.2013.1.2.083

50. Dakal TC, Kumar A, Majumdar RS, *et al*., 2016, Mechanistic basis of antimicrobial actions of silver nanoparticles. *Front Microbiol*, 7: 1831.

https://doi.org/10.3389/fmicb.2016.01831

51. Durán N, Fávaro WJ, Alborés S, *et al*., 2023. Biogenic silver nanoparticles capped with proteins: Timed knowledge and perspectives. *J Braz Chem Soc*, 34: 897–905.

https://doi.org/10.21577/0103-5053.20230062

52. Hamouda RA, Abd El-Mongy M, Eid KF, 2019, Comparative study between two red algae for biosynthesis silver nanoparticles capping by SDS: Insights of characterization and antibacterial activity. *Microb Pathog*, 129: 224–232.

https://doi.org/10.1016/j.micpath.2019.02.016

53. Khorrami S, Zarrabi A, Khaleghi M, *et al*., 2018, Selective cytotoxicity of green synthesized silver nanoparticles against the MCF-7 tumor cell line and their enhanced antioxidant and antimicrobial properties. *Int J Nanomedicine*, 13: 8013–8024.

https://doi.org/10.2147/IJN.S189295

54. Liao C, Li Y, Tjong SC, 2019, Bactericidal and cytotoxic properties of silver nanoparticles. *Int J Mole Sci*, 20(2): 449.

https://doi.org/10.3390/ijms20020449

ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

Association of *CCR2* gene polymorphism with chronic kidney disease in the North Indian population

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Abstract

Chronic kidney disease (CKD), characterized by decreased renal function, is associated with an underlying elevated renal inflammatory state. The chemokine receptor 2 (CCR2) mediates leukocyte chemoattraction in the initiation and amplification phases of renal inflammation. In this study, which involved 62 patients and 62 controls, we aim to elucidate the association between the *CCR2 G190A* (rs1799864) polymorphism and CKD. Polymerase chain reaction-restriction fragment length polymorphism technique-based single-nucleotide polymorphism genotyping of the *CCR2* gene (rs1799864) was used to assess the allele and genotype frequencies of *CCR2*. The Chi-square test was used to assess the potential association between *G190A* polymorphism and CKD, including its stages. The presence of the *CCR2 G190A* polymorphism was significantly associated with CKD. The results unveiled a significant difference in the genotype and allele frequency distribution of *CCR2 G190A* in CKD patients and control subjects. *CCR2* GA genotype ($P = 0.003$) and A allele ($P = 0.007$; odds ratio [OR] = 0.40; 95% confidence interval [CI] = 0.20 – 0.80) were found to be significantly associated with CKD. A significant association was identified between genotype and stage of CKD, with the GA genotype more common among end-stage renal disease (ESRD) (stage 5) and the GG genotype among non-ESRD (stages 1 – 4) patients ($P < 0.001$). The polymorphism was significantly associated with deteriorating renal function, as evidenced by elevated levels of serum urea, serum creatinine, and spot urine, alongside a decrease in serum calcium and estimated glomerular filtration rate. The study revealed that the GA genotype of *CCR2* is associated with CKD, ESRD, severe albuminuria, and renal dysfunction. However, no association was observed between the *CCR2* gene polymorphism and the causes of CKD in the North Indian population.

Keywords: Chronic kidney disease; End-stage renal disease; Chemokine receptor-2; Single-nucleotide polymorphism; Polymerase chain reaction-restriction fragment length polymorphism

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1. Introduction

Chronic kidney disease (CKD) is a leading public health problem worldwide[1] and affects between 8% and 16% of the global population. However, evidence suggests that it is

often under-recognized by both patients and clinicians[2,3]. Ironically, CKD prevalence is more commonly reported in low- and middle-income countries than in high-income countries[4]. CKD is defined as the presence of kidney damage or an estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2, persisting for 3 months or more, irrespective of the cause[5]. The progression of CKD leads to a loss in kidney function, ultimately resulting in the need for dialysis or renal replacement therapy. Given the poor prognosis associated with CKD, early diagnosis and disease screening are crucial for managing its prevalence. CKD frequently coexists with diabetes and/or hypertension, though other causes such as glomerulonephritis, infection, and environmental exposures are also prevalent in Asia, sub-Saharan Africa, and other developing countries[6]. Much like in Western countries, nearly half of the CKD cases in India are attributed to diabetes and hypertension[7]. Contemporary studies have reported a CKD prevalence rate of 17.5%[8,9], a figure driven by the rising incidence of diabetics and hypertension in the country. The prevalence of CKD in India exceeds the global average, highlighting the need for further studies to evaluate prognosis, markers, and associated comorbidities.

The pathophysiological mechanisms of CKD result in a progressive loss of renal function due to factors such as tubulointerstitial fibrosis, hypoxia-induced interstitial capillary damage, renal tubule destruction, and loss of functional nephrons. Locally produced chemokines exacerbate renal damage in CKD through profibrotic and inflammatory mechanisms. It is essential to interrupt the chemokine signal to reduce inflammation[10]. The recruitment of macrophages to the kidney in nephropathy is mostly dependent on the chemokine ligand 2/chemokine receptor 2 (CCL2/CCR2) signaling pathway. Treatment with the CCR2 antagonist RS504393 dramatically decreased infiltrating macrophages in diabetic mouse (db/db) mice, enhanced insulin resistance, and improved albuminuria, thereby mitigating renal injury[11]. Thus, for a deeper comprehension of the disease mechanism, it is essential to identify genetic variations in chemokines and their functional impact on disease status.

Polymorphisms in chemokine genes may cause interindividual differences in transcriptional control, resulting in a variable synthesis of pro-inflammatory molecules. Single-nucleotide polymorphisms (SNPs) in the *CCR2* gene have been implicated in various diseases in multiple studies, including CKD, diabetic nephropathy, immunoglobulin A nephropathy, and hypertension[10,12-14]. These studies collectively suggest that SNPs in the *CCR2* gene may play a role in CKD, potentially influencing inflammation and oxidative stress. However, further research is needed to fully understand the impact of these SNPs on CKD.

CCR2 is a chemokine receptor of the monocyte chemoattractant protein 1 (MCP1), a member of the chemotactic cytokines (CC) family of chemokines, primarily expressed on monocytes[15]. The CCR2 chemokine receptor mediates leukocyte chemoattraction during the initiation and amplification phases of renal inflammation[16]. The CCR2 protein consists of 374 amino acids, and the *CCR2*-V64I polymorphism is a transition mutation that changes valine to isoleucine at position 64 of the CCR2 receptor[17].

Recent findings underscore the therapeutic importance of chemokines in chronic renal failure, due to their pivotal role in disease pathogenesis[18] through CCL2, the ligand of the CCR2 receptor. CCL2 has been implicated as a key mediator of CKD in both human and animal models[19-22]. In addition, pharmacological inhibition of CCL2 has demonstrated efficacy in reducing chronic renal damage in lupus nephritis[23], improving podocyte function in diabetic nephropathy, and improving renal function in diabetic patients with albuminuria[24]. Given the importance of CCR2 and its ligand-mediated effect in kidney pathophysiology, the present case–control study aimed to investigate the association of *CCR2* gene polymorphism with susceptibility to CKD in the North Indian population.

2. Materials and methods

2.1. Study population

This case–control study involved 62 North Indian patients with CKD, 62.9% of whom were men. Subjects were recruited from the Department of Medicine at Era's Medical College and Hospital, Lucknow, India. The diagnosis of CKD was established based on eGFR and albuminuria, with eGFR calculated using the Cockcraft-Gault formula and serum creatinine levels. Further, classification of CKD followed the Kidney Disease Outcomes Quality Initiative (KDOQI) criteria for stages of CKD. Patients were divided according to eGFR into different stages: non-end-stage renal disease (non-ESRD) (grades 1 – 4, eGFR 15 – 90 mL/min/1.73 m^2) and end-stage renal disease (ESRD) stage (grade 5; eGFR < 15 mL/min/1.73 m^2)[25]. A total of 62 healthy unrelated individuals with normal renal function (56.5% of whom were men) from the same geographic location were included as controls, excluding those who suffered from acute kidney injury, cardiovascular disease, sepsis, or were critically ill. The study was approved by the Institutional Ethical Committee of Era's Lucknow Medical College and Hospital. This study was conducted in conformity with the Declaration of Helsinki, and informed consent was obtained from all subjects before sample collection. After obtaining written informed consent and a detailed history,

a 3-mL peripheral blood sample was obtained from all study participants in an EDTA vial. The blood samples were stored at −20°C in a refrigerator until DNA isolation. Clinical parameters associated with the *CCR2* SNP were assessed, including a complete blood count, serum levels of urea, creatinine, blood urea nitrogen, calcium, phosphorus, sodium, potassium, and uric acid. In addition, spot urine samples were collected for microalbumin and creatinine analysis. Radiological investigations, such as ultrasound, were conducted to assess kidney size.

2.2. Sample size

The sample size was calculated based on the odds ratio (OR) of the dominant A allele of *CCR2* in developing ESRD in the study population using Equation I[17]:

$$n = \frac{\left(z_\alpha + z_\beta\right)^2}{\left[ln\left(1-e\right)\right]^2}\left[\frac{1-p_1}{p_1} + \frac{1-p_2}{p_2}\right] \quad \text{(I)}$$

Where p_1 = 1/OR (OR = 32.4) and p_2 = 1.0 (the standard OR under the null hypothesis). The clinically considered difference in OR under the research hypothesis is e = 0.8. The type I error (α) was set at 5%, while the type II error (β) was set at 20%. The power of the study and the loss to follow-up values were 80% and 10%, respectively. Consequently, the sample size was calculated to be n = 73 in each group. However, due to restrictions imposed during the COVID-19 pandemic, only a sample size of 62 in each group was included.

2.2.1. DNA Extraction

DNA was extracted from whole blood samples using the QIAmp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol and stored at -20°C until use. The quality and quantity of DNA were assessed using a 1% agarose gel and a Nanodrop spectrophotometer (NanoDrop™ 2000/2000c Spectrophotometers, Thermo Fisher Scientific Inc., India).

2.2.2. Genotyping

The polymerase chain reaction (PCR) was used to amplify the polymorphic regions, and the restriction fragment length polymorphism (RFLP) technique was applied to detect the *CCR2* promoter SNP at the positions of −190 (G > A) (rs1799864). The PCR reaction was performed in a 20-µL reaction volume containing 50 ng genomic DNA, 10 µL PCR master mix (EmeraldAmp GT PCR Master Mix, Takara Bio Inc., India) containing 1 mmol/L $MgCl_2$, 100 mmol/L deoxynucleotide triphosphate (dNTP) and 0.5 U Taq polymerase (G-Biosciences, India), and 10 pmol of the following primers (Integrated DNA Technologies, California, USA):

Forward: 5'-CAT TGC AAT CCC AAA GAC CCA CTC -3'
Reverse: 5'-TTG GTT TTG TGG GCA ACA TGA TGG-3'

The PCR conditions were as follows: an initial denaturation step at 94°C for 5 min, followed by 33 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s, with a final extension at 72°C for 5 min. Cycling conditions were standardized on a conventional PCR machine (T100 Thermal cycler, Bio-Rad Laboratories, Inc, India). After successful amplification, the 173-bp PCR products of the *CCR2* gene were subjected to RFLP. RFLP digestion was performed in a 20-µL reaction mixture containing 2.5 U of BseJI (BsaBI) restriction enzyme (Thermo Fisher Scientific, Vilnius, Lithuania) and incubated at 65°C for 2 h. The digested products were examined and visualized using a gel documentation system (UVP DigiDoc-It Drawer, Analytik Jena GmbH + Co. KG, Germany) followed by 3% agarose gel electrophoresis. The undigested PCR product with 173 bp represented the G allele. The presence of the A allele was confirmed by visualizing two fragments of the digested PCR product with 149 bp and 24 bp (Figure 1).

The 3% agarose gel picture displays the PCR-RFLP product of the *CCR2* gene digested using the BseJI restriction enzyme. Lanes 1 (L1), 2 (L2), and 4 (L4) depict the AG genotype corresponding to bands of size 173 and 149 bp; lanes 3 (L3), 6 (L6), 7 (L7), and 8 (L8) indicate the GG genotype corresponding to bands of size 173 bp; lane 5 (L5) indicates a 100-bp ladder.

2.3. Statistical analysis

The data were analyzed using SPSS software version 23. Demographics, laboratory parameters, and the association of *CCR2* genotype with renal function parameters were evaluated through the Student's *t*-test for continuous variables and expressed as mean ± standard deviation (SD). Genotype and allele frequencies were compared between the ESRD patients and controls using the Chi-square (χ^2) test. $P < 0.05$ indicated a statistically significant difference.

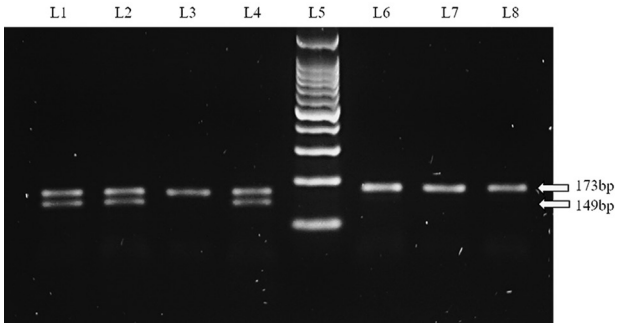

Figure 1. Polymerase chain reaction-restriction fragment length polymorphism image of *CCR2* gene (rs1799864) polymorphism.

3. Results

3.1. Heterogeneity analysis in case and control groups

This study involved 62 cases and 62 controls, and both groups were comparable for age and gender. Table 1 presents the comparison of demographic parameters between cases and controls. The majority of the cases of CKD had hypertension (91.9%) and diabetes (72.6%), while in non-CKD controls, only a few had comorbidities. On statistical comparison, a significant difference was observed between groups for diabetes and hypertension. Table 2 presents a comparison of comorbidities between the cases and the controls. Renal function parameters (urea, creatinine, eGFR, and spot urine) were significantly elevated in the cases (126.31 ± 38.71 mg/dL, 8.44 ± 3.24 mg/dL, 16.61 ± 7.30 mL/min/1.73 m^2, and 231.77 ± 156.25 mg/g, respectively) as compared to the controls (27.09 ± 5.18 mg/dL, 0.94 ± 0.22 mg/dL, 92.53 ± 23.27 mL/min/1.73 m^2, 15.95 ±6.59 mg/g). Serum calcium and hemoglobin were significantly lower in the cases (8.54 ± 0.73 mg/dL and 9.45 ± 1.76 g/dL, respectively) compared to the controls (9.08 ± 0.74 mg/dL and 13.23 ± 1.93 g/dL, respectively). However, serum albumin in the cases was 3.39 ± 2.94 mg/dL, which was comparable to the controls (3.77 ± 0.43 mg/dL). Table 3 presents the comparison of laboratory parameters between cases and controls.

Table 1. Comparison of demographic parameters between cases and controls

Parameter	Total (*N*=124)	Cases (*N*=62)		Controls (*N*=62)	
		n	%	*N*	%
Age					
≤20 years	4	2	3.2	2	3.2
21–30 years	17	10	16.1	7	11.3
31–40 years	22	11	17.7	11	17.7
41–50 years	17	9	14.5	8	12.9
51–60 years	29	14	22.6	15	24.2
61–70 years	24	10	16.1	14	22.6
≥71 years	11	6	9.7	5	8.1
		χ^2=1.380; *P*=0.967			
		Mean	**SD**	**Mean**	**SD**
		48.95	16.55	49.90	15.15
		t = −0.334; *P*=0.739			
Gender					
Female	50	23	37.1	27	43.5
Male	74	39	62.9	35	56.5
		χ^2=0.536; *P*=0.464			

Notes: Chi-square (χ^2) test was used to compare proportions between chronic kidney disease (CKD) cases and controls. Student's *t*-test is used to assess the mean age between CKD cases and controls. *P*<0.05 is considered significant. Abbreviation: SD: Standard deviation.

Table 2. Comparison of comorbidities between cases and controls

Comorbidities	Total	Cases		Controls		Chi-square test
		n	%	*n*	%	
Diabetes	67	45	72.6	22	35.5	χ^2=17.176; *P*<0.001
Hypertension	81	57	91.9	24	38.7	χ^2=38.770; *P*<0.001

Notes: Chi-square (χ^2) test was used to assess comorbidities between chronic kidney disease cases and controls. *P*<0.05 indicates a statistically significant difference.

3.2. Association of *CCR2 G190A* polymorphism with CKD

The majority of patients in both groups possess the GG genotype. However, a smaller proportion of subjects in the cases possessed the GG genotype compared to the control group (51.6% vs. 77.4%). A statistically significant difference was observed between the groups for *CCR2* polymorphism among cases and controls. The G allele was found to be prevalent (82.26%) in the total subjects enrolled in the study. However, a higher proportion of cases had an A allele (27.42%) as compared to the controls (11.29%). On statistical comparison, a significant association was observed between the A allele and CKD. The OR for the G allele was 0.40 with a confidence interval (CI) of 0.20–0.080. Table 4 presents a detailed comparison of genotype and allele frequencies of the *CCR2* gene (rs1799864) between cases and controls. No difference was observed between *CCR2* gene polymorphism and the cause of CKD. Table 5 presents the association of the *CCR2* genotype with the causes of CKD. Elevated renal function parameters, except eGFR (urea, creatinine, and spot urine), were significantly associated with the GA genotype of the *CCR2* gene. Table 6 presents the association of the *CCR2* genotype with the renal function parameters. A statistically significant association was observed for the GA genotype of the *CCR2* gene with ESRD. Table 7 presents the association of the *CCR2* gene with stages of CKD.

4. Discussion

Chronic kidney disease is a major public health concern and is commonly attributed to factors such as diabetes, hypertension, nephrotoxic drugs, and glomerulonephritis. The identification of numerous genes associated with monogenic kidney illnesses with classical inheritance patterns, as well as genes for complex kidney diseases that manifest in conjunction with environmental variables, is feasible. Genetic discoveries are increasingly being utilized

Table 3. Comparison of laboratory parameters between cases and controls

Parameters	Cases (*N*=62)		Controls (*N*=62)		Student's *t*-test	
	Mean	SD	Mean	SD	*T*	*P*
Urea (mg/dL)	126.31	38.71	27.09	5.18	20.004	<0.001
Creatinine (mg/dL)	8.44	3.24	0.94	0.22	18.196	<0.001
eGFR (mL/min/1.73 m^2)	16.613	7.30	92.53	23.27	−24.507	<0.001
Spot urine (mg/g)	231.77	156.25	15.95	6.59	10.867	<0.001
Calcium (mg/dL)	8.54	0.73	9.08	0.74	−4.052	<0.001
Albumin (mg/dL)	3.39	2.94	3.77	0.43	−0.997	0.321
Hemoglobin (g/dL)	9.45	1.76	13.23	1.93	−11.398	<0.001

Notes: Student's *t*-test was used for continuous variables. $P<0.05$ indicates a statistically significant difference. Abbreviations: eGFR: Estimated glomerular filtration rate; SD: Standard deviation.

Table 4. Comparison of genotype and allele frequencies of *CCR2* gene (rs1799864) between cases and controls

Variable	Total (*N*=124)		Cases (*N*=62)		Controls (*N*=62)	
	n	%	*n*	%	*n*	%
Genotype						
GA	44	35.48	30	48.4	14	22.6
GG	80	64.52	32	51.6	48	77.4
AA	0	0	0	0.0	0	0.0
			χ^2=9.018; *P*=0.003			
Allele						
G	204	82.26	94	72.58	110	88.71
A	44	17.74	30	27.42	14	11.29
			χ^2=7.073; *P*=0.007			

Notes: Chi-square (χ^2) test was used to compare the *CCR2* genotype between chronic kidney disease cases and controls. $P<0.05$ indicates a statistically significant difference.

to guide the clinical care of nephropathies, leading to improvements in disease surveillance, drug selection, diagnostic accuracy, and family counseling. Accurate interpretation of genetic data is necessary for optimizing all of these processes[26]. In the same context, the present study aimed to investigate the association of the *CCR2* gene polymorphism with CKD. For this purpose, 62 patients with CKD (cases) and 62 age- and gender-matched non-CKD patients (controls) were enrolled in the study.

In the present study on *CCR2* polymorphism, the GG genotype was more prevalent in controls compared to cases (77.4% vs. 51.6%), whereas the GA genotype was more prevalent in cases (48.4%) compared to the controls (22.6%). Moreover, the G allele was more prevalent in controls (88.71%) compared to cases (72.58%), while cases expressed the A allele (27.42%) more frequently than the controls (11.29%). The *CCR2* GA genotype and A allele were associated with the occurrence of CKD and deterioration of renal functions, indicated by elevated levels of urea, creatinine, spot urine, and decreased levels of calcium and eGFR. Similar findings were observed by Sezgin *et al.*, where the frequency of the *CCR2* GA genotype was higher in cases, and the GG genotype was higher in controls. The A allele was more frequent in cases, and the G allele was more frequent in controls. The results were statistically significant and consistent with the present study[16]. Nakajima *et al.* reported that, although *CCR2* was not significantly associated with kidney disease, the frequency of the A allele was higher in patients with impaired kidneys[12]. Similarly, in the present study, the frequency of the A allele was observed to be higher in CKD patients (27.42%) than in non-CKD subjects (11.29%). In another study by Elghoroury *et al.*, the frequency of the A allele was significantly higher among children with ESRD. Moreover, they reported significantly higher frequencies of the GA+AA genotype among transplantation, hemodialysis, and ESRD patients, while the GG genotype was more prevalent in controls[17].

Beyond alterations in the nucleotide sequence, epigenetic molecular mechanisms may play a critical role in the progression of renal disease through inflammation. Epigenetic mechanisms include DNA methylation, histone protein modifications, and RNA interference. Epigenetic regulation of transcription plays a crucial role in normal physiological development and also in pathological conditions. For example, abnormal DNA methylation is linked to insulin resistance, inflammation, and immunological dysfunction. RNA interference may significantly contribute to the development of kidney disease, given the essential role of microRNAs in maintaining glomerular homeostasis. Epithelial-mesenchymal transition and subsequent renal tissue fibrosis are controlled by epigenetic changes. In CKD, epimutations might occur due to uremic toxins, oxidative stress, inflammation, and hyperhomocysteinemia. Due

Table 5. Association of *CCR2* genotype with the causes of chronic kidney disease

Causes	Genotype				Chi-square test
	GA		GG		
	n	%	*n*	%	
Diabetic nephropathy (*N*=40)	21	70.0	19	59.4	χ^2=1.400; *P*=0.706
Autosomal dominant polycystic kidney disease (ADPKD) (*N*=2)	1	3.3	1	3.1	
Glomerulonephritis (*N*=7)	2	6.7	5	15.6	
Hypertensive nephrosclerosis (*N*=13)	6	20.0	7	21.9	

Notes: The Chi-square (χ^2) test was used to determine the association of the *CCR2* genotype with causes of CKD. *P*<0.05 indicates a statistically significant difference.

Table 6. Association of *CCR2* genotype with renal function parameters

Parameters	GA		GG		Student's *t*-test	
	Mean	SD	Mean	SD	*t*	*P*
Urea (mg/dL)	104.82	69.04	64.28	45.83	3.859	< 0.001
Creatinine (mg/dL)	6.96	5.43	3.69	3.46	4.043	< 0.001
eGFR (mL/min/1.73 m^2)	45.00	45.66	58.80	39.53	−1.708	0.090
Spot urine (mg/g)	212.18	191.86	84.84	116.13	4.559	< 0.001

Notes: Student's *t*-test was used to determine the association of the *CCR2* genotype with renal function parameters. *P*<0.05 indicates a statistically significant difference. Abbreviations: eGFR: Estimated glomerular filtration rate; SD: Standard deviation.

Table 7. Association of *CCR2* gene (rs1799864) with the stages of chronic kidney disease (CKD) in the CKD group

Stage (*N*=62)	GA		GG		Chi-square test
	n	%	*n*	%	
Non-ESRD (Grades 1–4) (*n*=34)	9	30.0	25	78.1	χ^2=14.480; *P*<0.001
ESRD (Grade 5) (*n*=28)	21	70.0	7	21.9	

Notes: The Chi-square (χ^2) test was used to determine the association of the *CCR2* genotype with the stages of CKD. *P*<0.05 indicates a statistically significant difference. Abbreviation: ESRD: End-stage renal disease.

to the reversible nature of epigenetic modifications, there is a potential to halt or even reverse the disease process through targeted therapy[27].

The novelty of this study lies in the evaluation of the association of *CCR2* with causes, age of onset, and duration of CKD, which were discovered to be comparable. The present study also explored the correlation of the stage of renal disease with *CCR2*, a facet with limited findings in contemporary studies, adding novelty to our research. However, a number of shortcomings exist in this study. First, the sample size might be considered small, with only 62 CKD patients and 62 non-CKD controls. Second, selection bias was inevitable. Third, our investigation was limited to the North Indian population and did not encompass all regions. Therefore, to validate our findings, further research with a larger sample size and a more diverse range of racial backgrounds is required. Ongoing experiments in our laboratory aim to identify the role of polymorphisms in various genes and their implications for genetic predisposition to CKD in a larger patient population, enhancing our understanding of the genetic basis underlying CKD.

5. Conclusion

The findings of the present study suggest a significant association between the *CCR2* GA genotype and CKD, along with deteriorating renal function and stages of renal disease. While the G allele was more frequently observed overall, the A allele demonstrated a significant association with CKD. Elevated levels of parameters indicating deteriorating renal function (urea, creatinine, and spot urine) were significantly associated with the GA genotype of *CCR2*. These findings indicate that the GA genotype of *CCR2* is associated with CKD, ESRD, severe albuminuria, and renal dysfunction, but not with the causes or duration of CKD. However, the age of onset, duration, and causes of CKD demonstrated no association with *CCR2* polymorphism in the North Indian population.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Devendra Kumar, Syed Tasleem Raza
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Investigation: Aseem Yadav
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Writing—original draft: Saliha Rizvi
Writing—review & editing: Saliha Rizvi, Farheen Khan

Ethics approval and consent to participate

This study was performed in accordance with the Declaration of Helsinki. The protocol and procedures employed were reviewed and approved by the Institutional Ethical Committee, Era's Lucknow Medical College and Hospital, Lucknow, India (Approval no.: Rcell EC/2020/78). A written informed consent was obtained from all the subjects before sample collection to participate in the study.

Consent for publication

The authors affirm that human research participants provided informed consent for publication of their data.

Availability of data

Data used in this work are available from the corresponding author on reasonable request.

References

1. Sarnak MJ, Levey AS, Schoolwerth AC, *et al*., 2003, Kidney disease as a risk factor for development of cardiovascular disease: A statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Circulation*, 108(17): 2154–2169.

   https://doi.org/10.1161/01.CIR.0000095676.90936.80

2. Coresh J, Selvin E, Stevens LA, *et al*., 2007, Prevalence of chronic kidney disease in the United States. *JAMA*, 298(17): 2038–2047.

   https://doi.org/10.1001/jama.298.17.2038

3. Plantinga LC, Boulware LE, Coresh J, *et al*., 2008, Patient awareness of chronic kidney disease: Trends and predictors. *Arch Intern Med*, 168(20): 2268–2275.

   https://doi.org/10.1001/archinte.168.20.2268

4. Mills KT, Xu Y, Zhang W, *et al*., 2015, A systematic analysis of worldwide population-based data on the global burden of chronic kidney disease in 2010. *Kidney Int*, 88(5): 950–957.

   https://doi.org/10.1038/ki.2015.230

5. Chapter 1: Definition and classification of CKD. *Kidney Int Suppl* (*2011*), 2013, 3(1): 19–62.

   https://doi.org/10.1038/kisup.2012.64

6. Jha V, Garcia-Garcia G, Iseki K, *et al*., 2013, Chronic kidney disease: Global dimension and perspectives. *Lancet*, 382(9888): 260–272.

   https://doi.org/10.1016/S0140-6736(13)60687-X

7. Rajapurkar MM, John GT, Kirpalani AL, *et al*., 2012, What do we know about chronic kidney disease in India: First report of the Indian CKD registry. *BMC Nephrol*, 13: 10.

   https://doi.org/10.1186/1471-2369-13-10

8. Singh AK, Farag YM, Mittal BV, *et al*., 2013, Epidemiology and risk factors of chronic kidney disease in India - results from the SEEK (Screening and Early Evaluation of Kidney Disease) study. *BMC Nephrol*, 14: 114.

   https://doi.org/10.1186/1471-2369-14-114

9. Varma PP, 2015, Prevalence of chronic kidney disease in India - Where are we heading? *Indian J Nephrol*, 25(3): 133–135.

10. Alkady MM, Abdel-Messeih PL, El-Fishawy HS, *et al*., 2020, Evaluation of chemokine receptor 2 polymorphism in patients with end-stage renal disease. *J Egypt Soc Nephrol Transpl*, 20(3): 151–156.

    https://doi.org/10.4103/jesnt.jesnt_2_20

11. Li HD, You YK, Shao BY, *et al*., 2022, Roles and crosstalks of macrophages in diabetic nephropathy. *Front Immunol*, 13: 1015142.

    https://doi.org/10.3389/fimmu.2022.1015142

12. Nakajima K, Tanaka Y, Nomiyama T, *et al*., 2002, Chemokine receptor genotype is associated with diabetic nephropathy in Japanese with type 2 diabetes. *Diabetes*, 51(1): 238–242.

    https://doi.org/10.2337/diabetes.51.1.238

13. Gao J, Liu X, Wei L, *et al*., 2016, Genetic variants of MCP-1 and CCR2 genes and IgA nephropathy risk. *Oncotarget*, 7: 77950–77957.

    https://doi.org/10.18632/oncotarget.12847

14. Zhang M, Ardlie K, Wacholder S, *et al*., 2006, Genetic variations in CC chemokine receptors and hypertension. *Am J Hypertens*, 19(1): 67–72.

    https://doi.org/10.1016/j.amjhyper.2005.06.025

15. Kitagawa K, Wada T, Furuichi K, *et al*., 2004, Blockade of CCR2 ameliorates progressive fibrosis in kidney. *Am J Pathol*, 165(1): 237–246.

    https://doi.org/10.1016/S0002-9440(10)63292-0

16. Sezgin I, Koksal B, Bagci G, *et al*., 2011, CCR2 polymorphism in chronic renal failure patients requiring long-term hemodialysis. *Intern Med*, 50(21): 2457–2461.

    https://doi.org/10.2169/internalmedicine.50.5119

17. Elghoroury EA, Elshamaa MF, Fadel FI, *et al*., 2018, CCR2 V64I genotyping: Impact on end stage renal disease development, progression and renal transplantation outcome. *JIBPS*, 5(4): 5–11.

18. Anders HJ, Vielhauer V, Schlöndorff D, 2003, Chemokines and chemokine receptors are involved in the resolution or progression of renal disease. *Kidney Int*, 63(2): 401–415.

https://doi.org/10.1046/j.1523-1755.2003.00750.x

19. Kashyap S, Warner GM, Hartono SP, *et al*., 2016, Blockade of CCR2 reduces macrophage influx and development of chronic renal damage in murine renovascular hypertension. *Am J Physiol Renal Physiol*, 310(5): F372–F384.

https://doi.org/10.1152/ajprenal.00131.2015

20. Matsunaga N, Ikeda E, Kakimoto K, *et al*., 2016, Inhibition of G0/G1 switch 2 ameliorates renal inflammation in chronic kidney disease. *eBioMedicine*, 13: 262–273.

https://doi.org/10.1016/j.ebiom.2016.10.008

21. Eardley KS, Zehnder D, Quinkler M, *et al*., 2006, The relationship between albuminuria, MCP-1/CCL2, and interstitial macrophages in chronic kidney disease. *Kidney Int*, 69(7): 1189–1197.

https://doi.org/10.1038/sj.ki.5000212

22. Tesch GH, 2008, MCP-1/CCL2: A new diagnostic marker and therapeutic target for progressive renal injury in diabetic nephropathy. *Am J Physiol Renal Physiol*, 294(4): F697–F701.

https://doi.org/10.1152/ajprenal.00016.2008

23. Kulkarni O, Pawar RD, Purschke W, *et al*., 2007, Spiegelmer inhibition of CCL2/MCP-1 ameliorates lupus nephritis in MRL-(Fas)lpr mice. *J Am Soc Nephrol*, 18(8): 2350–2358.

https://doi.org/10.1681/ASN.2006121348

24. Boels MGS, Koudijs A, Avramut MC, *et al*., 2017, Systemic monocyte chemotactic protein-1 inhibition modifies renal macrophages and restores glomerular endothelial glycocalyx and barrier function in diabetic nephropathy. *Am J Pathol*, 187(11): 2430–2440.

https://doi.org/10.1016/j.ajpath.2017.07.020

25. National Kidney Foundation, 2002, K/DOQI clinical practice guidelines for chronic kidney disease: Evaluation, classification, and stratification. *Am J Kidney Dis*, 39: S1–S266.

26. Köttgen A, Gall ECL, Halbritter J, *et al*., 2022, Genetics in chronic kidney disease: Conclusions from a kidney disease: Improving Global Outcomes (KDIGO) controversies conference. *Kidney Int*, 101(6): 1126–1141.

https://doi.org/10.1016/j.kint.2022.03.019

27. Dwivedi RS, Herman JG, McCaffrey TA, *et al*., Beyond genetics: Epigenetic code in chronic kidney disease. *Kidney Int*, 79(1): 23–32.

https://doi.org/10.1038/ki.2010.335

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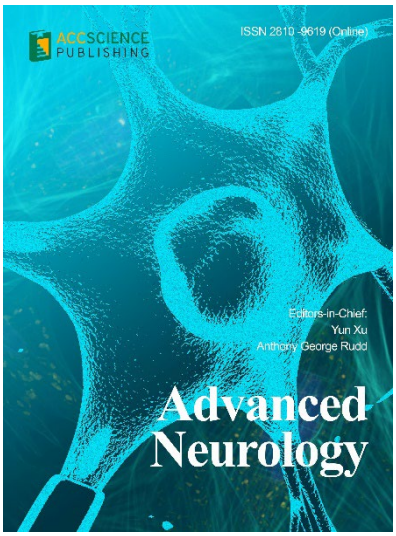

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- Neurodegenerative disease
- Cerebrovascular disease
- Epilepsy and movement disorders
- Neuroimmune disease
- Neurological infections
- Muscle disease
- Molecular and cellular neuroscience
- Systems neuroscience
- Cognitive neuroscience
- Computational modeling of nervous system

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