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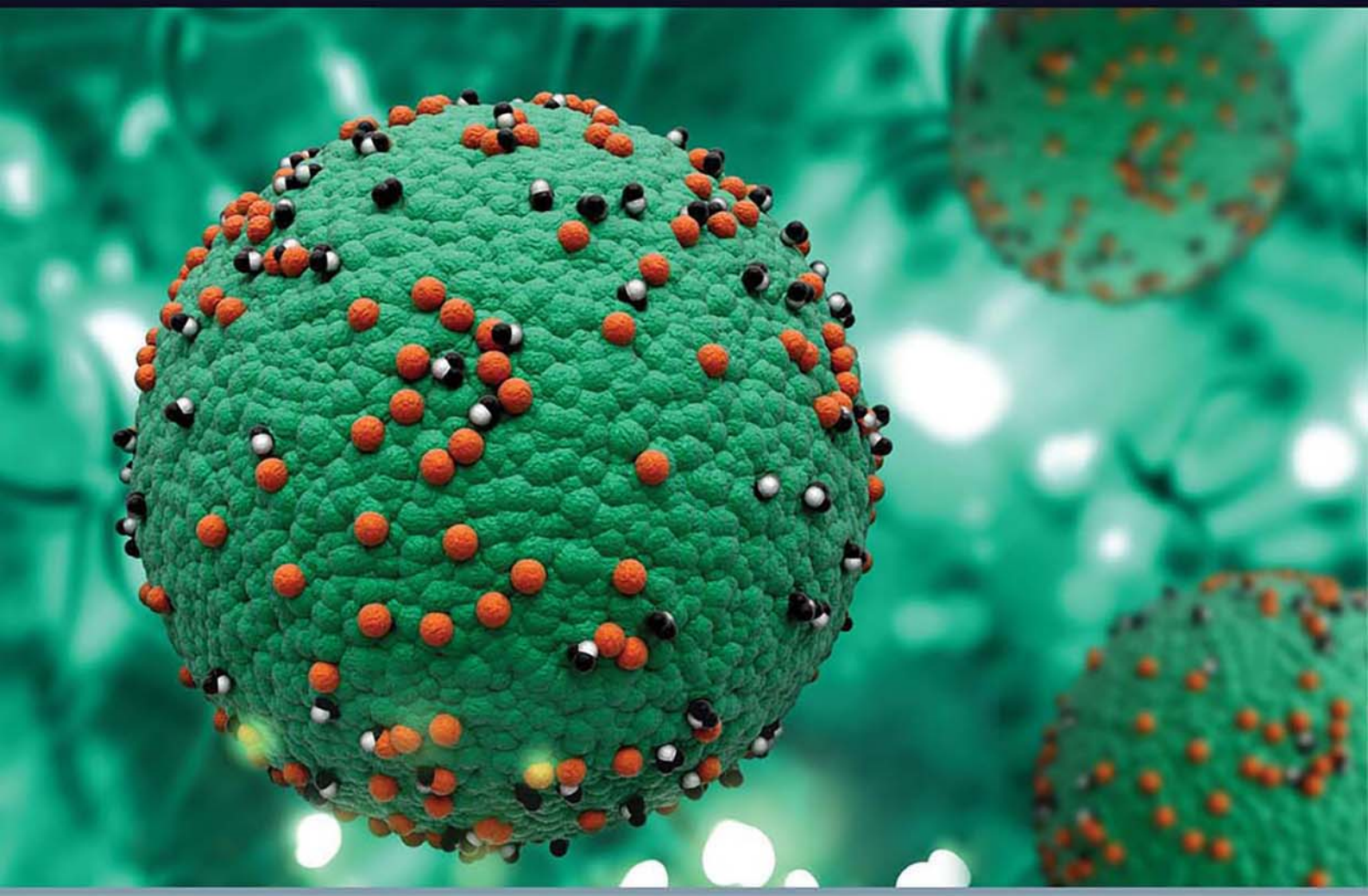

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Contact Information
Tel: (98)2188985291-3
Fax: (98)2189775181
Email: Editor@pmjournal.ir
Address: No. 2, Italia Street, Tehran, Iran
Poqal code : 1416673744

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The Evolving Landscape of Drug Resistance: From Mechanisms to Therapeutic Strategies

Yousef Roosta[1,*], **Neda Abedi**[2]

[1]Department of Internal Medicine, Faculty of Medicine, University of Medical Sciences, Urmia, Iran.
[2]Department of Microbiology, North Branch of Azad University, Tehran, Iran.

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Corresponding author:
Yousef Roosta,Department of Internal Medicine, Faculty of Medicine, University of Medical Sciences, Urmia, Iran. Email: yroosta@gmail.com.

ABSTRACT

Drug resistance is a major obstacle in the effective treatment of cancer, severely impacting patient outcomes and complicating therapeutic strategies. The development of resistance is multifactorial, involving a combination of genetic and epigenetic changes within cancer cells, alterations in drug metabolism, increased DNA repair mechanisms, overexpression of drug efflux pumps, and complex interactions with the tumor microenvironment. These factors work synergistically to render traditional chemotherapy and targeted therapies less effective over time.

Recent advances in molecular biology, particularly next-generation sequencing and the CRISPR-Cas9 gene-editing tool have significantly enhanced our understanding of the underlying mechanisms driving resistance. These technologies have enabled researchers to identify novel genetic mutations and signaling pathways that cancer cells exploit to evade treatment, offering new potential targets for therapeutic intervention. Additionally, the dynamic role of the tumor microenvironment, including immune cells, stromal cells, and extracellular matrix components, has emerged as a key factor influencing drug resistance, further complicating treatment strategies.

To address these challenges, several innovative therapeutic approaches are being explored. Combination therapies, which involve the use of multiple drugs targeting different pathways simultaneously, hold promise in overcoming resistance by attacking cancer cells from multiple fronts. Immunotherapy, which harnesses the body's immune system to target cancer cells, is also showing significant potential in resistant cancers. Furthermore, nanomedicine, which uses nanoparticles to deliver drugs directly to tumors, may improve drug efficacy and minimize resistance.

Despite these advancements, much remains to be done. Ongoing research focused on identifying reliable biomarkers, developing personalized medicine approaches, and understanding the intricate relationship between cancer cells and their microenvironment is essential. This review aims to provide a comprehensive overview of the current state of knowledge regarding drug resistance in cancer, emerging therapeutic strategies, and future research directions in this critical field.

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INTRODUCTION

Cancer is a complicated condition defined by the uncontrolled proliferation and dissemination of aberrant cells. It is caused by damage to DNA, the genetic material that regulates cell activity and development (1, 2). Mutations in DNA may arise in a variety of genes, such as oncogenes (which drive cell development) and tumor suppressor genes. Mutations

in these genes allow cells to develop unrestrained, creating tumors that may invade neighboring tissues and spread to distant regions (3). Cancer has several origins, including genetic, environmental, and lifestyle factors. Genetic alterations may be passed down from parents, raising a person's vulnerability to certain malignancies. Environmental factors including tobacco smoke, asbestos, and UV radiation may affect DNA and contribute to the occurrence of cancer (4). Diet, physical exercise, and alcohol intake all contribute significantly to cancer risk.

Cancer therapy has improved tremendously, providing a variety of alternatives based on the kind and stage of the disease (5). Common methods involve surgery to eliminate malignant tissue, radiation treatment to kill cancerous cells with high-energy beams, chemotherapy to utilize medications that target fast-proliferating cells, and tailored therapies to tackle particular weaknesses in cancer cells (6). Immunotherapy, which uses the body's immune system to combat cancer, is an increasingly essential therapeutic option. However, cancer therapy presents several obstacles (7). Many therapies, although effective against cancer cells, may harm healthy tissues, resulting in symptoms such as tiredness, nausea, and hair loss. Drug resistance is another key barrier since cancer cells might develop methods to avoid the effects of therapies (8).

Drug resistance is a serious impediment to effective cancer therapy, often causing disease recurrence and worse patient outcomes. Cancer cells, owing to their intrinsic genetic instability and fast multiplication, might develop methods to resist the damaging impacts of anticancer treatments (9). This resistance can manifest in a variety of ways, including decreased drug absorption by the cell, increased drug efflux (pushing the drug out of the cell), modifications in the targeted pathway (so the drug no longer connects successfully), stimulation of substitute signaling networks that bypass the drug's intended operation, and improved DNA repair processes that counteract the DNA damage caused by some chemotherapies (10). Drug resistance develops via a complicated process that is influenced by selection pressure. When malignant cells undergo exposure to a treatment, those cells that develop resistance responses have a survival advantage and are more likely to multiply, ultimately becoming the majority group within the tumor (11).

Several pathways are associated with the emergence of drug resistance. Genetic changes may occur spontaneously or be caused by the medicine, resulting in changed proteins that impact drug action or metabolism (12). Epigenetic alterations, which modify gene expression without altering the DNA sequence, may also have an impact. Modifications in DNA methylation or histone changes, for instance, might influence gene expression related to drug metabolism or cell viability. In addition, the tumor environment may lead to medication resistance (13). Hypoxia (low oxygen concentrations), nutritional restriction, and the existence of specific immune cells may all contribute to a selection pressure that promotes the persistence of drug-resistant cells (14). Addressing the intricate interaction of these processes is critical for creating methods to avoid or overcome drug resistance and enhance cancer treatment results. This study investigates the complicated processes behind medication resistance in cancer, a key hindrance to effective therapy, as well as the problems it presents. Furthermore, it looks into new alternative treatment tactics for overcoming resistance and increasing patient results (15).

Mechanisms involved in drug resistance

Efflux pumps

Efflux pumps, especially those from the ATP-binding cassette (ABC) transporter superfamily, are critical in the emergence of multidrug resistance (MDR) in cancer cells (16). These pumps are transmembrane proteins that actively transport a diverse range of materials, notably various chemotherapeutic medicines, out of the cell. Efflux pumps reduce the intracellular concentration of these medicines, limiting their capacity to communicate with their cell targets and producing cytotoxic consequences (17).

This lower dosage enables tumor cells to survive and grow in the presence of chemotherapeutic resulting in chemotherapy failure. Numerous ABC transporters, including P-glycoprotein (P-gp/ABCB1), multidrug resistance-associated protein 1 (MRP1/ABCC1), and breast cancer resistance protein (BCRP/ABCG2), have been extensively researched and linked to MDR in diverse kinds of cancer (18). The increased expression of these efflux pumps is common in drug-resistant malignancies, and it is directly related to decreased drug efficacy and a poorer prognosis for patients. Additional resistance mechanisms, including changed drug targets or increased DNA repair, may work along with efflux pump activity to increase drug resistance (19). Recognizing the individual efflux pumps implicated in a given cancer type, as well as the variables that influence their expression and activity, is critical for devising ways to avoid MDR and increase cancer treatment efficacy (20).

Epigenetic modifications

Epigenetic changes, heritable variations in gene expression that do not entail modifications to the DNA sequence itself, play a crucial role in the establishment and maintenance of treatment resistance in cancerous cells (21). These alterations, which include DNA methylation, histone modifications, and changes in microRNA expression, may impact the expression

of genes that regulate drug metabolism, therapeutic targets, cell survival, and DNA repair, resulting in diminished medication effectiveness (22). For instance, hypermethylation of a tumor suppressor gene's promoter region might quiet its expression, eliminating a major barrier to cell proliferation and possibly granting resistance to treatments that target quickly expanding cells (23). On the other hand, hypomethylation of oncogenes may result in their overexpression, which promotes cell proliferation and treatment resistance. These epigenetic changes may be reversible, presenting prospective therapeutic options to restore medication sensitivity (24).

One extensively investigated instance of epigenetic regulation in drug resistance is the gene that encodes P-glycoprotein (P-gp), a notable efflux pump that leads to multidrug resistance. Elevated P-gp expression may efficiently pump chemotherapy medicines out of tumor cells, lowering their intracellular concentration and efficacy (25). Epigenetic changes, like DNA hypomethylation in the P-gp promoter region, may boost P-gp expression and develop drug resistance (26). Likewise, histone changes, such as enhanced histone acetylation, may promote P-gp gene transcription. Knowing the particular epigenetic alterations that control drug resistance mechanisms such as P-gp expression is critical for creating innovative treatment techniques to reverse these changes and restore drug sensitivity in malignant cells (27). These tactics may include the application of epigenetic medicines, like DNA methyltransferase inhibitors or histone deacetylase inhibitors, to remodel cancer cells' epigenomes and make them more receptive to chemotherapy (28).

Tumor microenvironment

The tumor microenvironment (TME), a complicated and ever-changing ecology around cancer cells, serves a vital role in cancer growth, metastasis, and, crucially, the emergence of treatment resistance (29). It encompasses some cellular and non-cellular elements, involving fibroblasts, immunological cells, endothelial cells (lining blood vessels), and extracellular matrix (ECM) constituents (30). This sophisticated network of connections has the potential to greatly alter cancer cell activity by supplying growth factors, viability signals, and protection from treatments (31). The TME may exert selective pressure on drug-resistant tumor cells, failing to treat. For example, the TME may be hypoxic, nutrient-deprived, or acidic, all of which might generate or choose tumor cells with improved drug resistance pathways (32). Likewise, physical characteristics of the TME, like thick ECM or aberrant blood vessel development, might impede medication delivery to the tumor site, hence lowering the drug's therapeutic effectiveness (33).

Different cell types in the TME are associated with drug resistance. Cancer-associated fibroblasts (CAFs), which frequently appear in the TME, may release growth hormones and ECM components that help tumor cells survive and fight drugs (34). They may also alter the ECM, forming a physical barrier that prevents drug entry. Immune cells, which are perfectly designed to kill cancerous cells, might paradoxically lead to treatment resistance (35). Tumor-associated macrophages (TAMs), for example, may adopt a pro-tumor character by secreting substances that promote tumor cell proliferation, angiogenesis (new blood vessel creation), and treatment resistance (36). Myeloid-derived suppressor cells (MDSCs) are another kind of immune cell that may inhibit anti-tumor response and cause treatment resistance via a variety of methods (37). Endothelial cells, which line blood arteries, can also lead to medication resistance by restricting drug delivery to tumors or supporting the survival of tumor cells that have spread to distant areas (38). The relationship between cancerous cells and the many cell types within the TME is complicated and bidirectional. Cancer cells may affect the activity of stromal cells, which can then offer assistance and defense to cancer cells, therefore developing treatment resistance (39). Knowing the intricate interactions that occur inside the TME is critical for creating innovative treatment techniques that target not just cancer cells but also supporting cells and the surrounding environment in general (40). Interfering with the TME's protective function, for example, by attacking CAFs, TAMs, or the ECM, may increase drug delivery and restore drug sensitivity in tumor cells, resulting in improved cancer therapy (41).

DNA repair pathways

DNA repair processes are key biological systems that preserve the integrity of the genome by identifying and fixing different forms of DNA damage, such as single-strand breaks, double-strand breaks, base alterations, and DNA crosslinks (42). These pathways rely on an intricate structure of proteins to identify DNA damage, activate repair processes, and restore the original DNA sequence. There are many unique DNA repair mechanisms, each specialized for fixing certain kinds of DNA damage (43). For example, nucleotide excision repair (44) eliminates large DNA adducts caused by UV radiation or chemical carcinogens, whereas base excision repair (BER) addresses destroyed or altered bases (45). The homologous recombination (HR) and non-homologous end joining (NHEJ) processes are crucial for mending double-strand breaks, which are especially harmful since they may lead to chromosomal instability and cell death. The capability and accuracy of DNA repair are critical for proper cell function and survival, and

abnormalities in these pathways may lead to higher mutation frequencies and cancer formation (46).

Cancer cells, although frequently described by genomic instability, may also demonstrate improved DNA repair capability as a strategy of resistance to DNA-damaging treatments, such like chemotherapy and radiation (47). These medicines produce DNA damage in cancer cells, prompting cell death by apoptosis. Yet, tumor cells with increased DNA repair function can effectively repair this damage, enabling them to survive and multiply despite therapy (48). This improved repair capability may occur via a variety of methods, such as increased production of DNA repair proteins, overexpression of certain repair pathways, or changes in the signaling pathways that control DNA repair (49). Elevated expression of ERCC1, a DNA repair protein, has been linked to resistance to platinum-based chemotherapies that cause DNA cross-links. Comparably overexpression of the HR pathway may provide resistance to ionizing radiation, which causes double-strand breaks (50).

The connection between DNA repair processes and drug resistance is complicated and multifaceted. Although improved DNA repair can protect tumor cells from the toxic properties of DNA-damaging therapies, it can also make them more susceptible to other kinds of therapies (51). For example, tumors cells with errors in specific DNA repair pathways, like BRCA1/2 in the HR pathway, can be more susceptible to PARP inhibitors, which block single-strand break repair (52). This synthetic lethality technique takes advantage of a defect in one DNA repair mechanism to make cancer cells more susceptible to blockage of another process (53). In addition, the tumor microenvironment might affect DNA repair ability. Hypoxia, for instance, may cause DNA damage and alter the expression of DNA repair proteins. Addressing the complex interaction of DNA repair, drug resistance, and the tumor environment is critical for designing customized cancer treatments that target particular tumor susceptibility (54).

Drug Resistance: A Challenge in Cancer Therapy

Drug resistance dramatically influences cancer therapy across many malignancies, reducing therapeutic effectiveness and influencing patient results. Resistance may turn a curable malignancy into a potentially fatal illness, restricting therapy choices and often resulting in disease progression and recurrence (55). This problem emerges variably among cancer types, mirroring the varied causes of resistance and therapies used. Drug resistance is a significant issue in hematological malignancies like acute myeloid leukemia (56, 57). AML cells may exhibit resistance to chemotherapy via a variety of processes, such as raised drug efflux via ABC transporters, mutations in drug targets like the FLT3 receptor tyrosine kinase, and changes in apoptotic routes (58). These mechanisms of resistance frequently result in relapse, necessitating more intensive and possibly less efficient salvaging therapies. Similarly, mutations in the BCR-ABL1 fusion gene, which drives chronic myeloid leukemia (CML), may cause resistance to tyrosine kinase inhibitors (TKIs) such as imatinib. These mutations could hinder the TKI from binding efficiently, enabling leukemic cells to grow uncontrolled (59).

Solid cancers also confront considerable issues with treatment resistance. In lung cancer, resistance to EGFR inhibitors, including gefitinib and erlotinib, usually develops owing to secondary mutations in the EGFR gene, most notably the T790M mutation (60). This mutation modifies the EGFR protein, rendering it insensitive to the inhibitor. Likewise, in breast cancer, resistance to HER2-targeted treatments such as trastuzumab may develop via a variety of processes, such as a higher level of alternative growth factor receptors, activation of downstream signaling pathways that bypass HER2, and loss of PTEN, a tumor suppression gene (61). Melanoma resistance to BRAF inhibitors, such as vemurafenib and dabrafenib, targeting the BRAF V600E mutation, may appear via reactivation of the MAPK system, either through mutations in other elements of the pathway or through activation of bypass signaling loops (62). Colorectal cancer is another instance of how drug resistance has an important effect on controlling patients. Resistance to treatment strategies like FOLFOX or FOLFIRI may develop via a variety of pathways, involving increased production of DNA repair enzymes, modifications to drug metabolism, and alterations in the tumor microenvironment (63). Resistance to targeted therapy, such as EGFR inhibitors (Cetuximab, Panitumumab) or VEGF inhibitors (bevacizumab), may also emerge via changes in downstream signaling pathways, like KRAS or NRAS, or by activation of alternative angiogenic variables (64). Platinum resistance in ovarian cancer is a significant clinical issue. Mechanisms involve enhanced DNA repair, altered drug delivery, and changes in glutathione levels. This resistance generally leads to disease recurrence and a worse prognosis (65). The emergence of medication resistance has a significant influence on patient outcomes. It often entails the application of more harmful and ineffective medicines, which leads to higher side effects and a worse quality of life. Furthermore, medication resistance might restrict treatment choices, making illness management challenging and eventually leading to lower survival rates (66). Addressing medication resistance requires continued study into the underlying processes, the development of innovative therapeutic tactics, and the deployment of personalized treatment methods that customize therapy to the unique features of every patient's tumor (67).

Molecular insights into drug resistance: advancing cancer therapy

Recent advancements in molecular biology have transformed our knowledge of drug resistance pathways in cancer, presenting tremendous prospects for creating more efficient treatments (68). Technologies like next-generation sequencing provide thorough genomic profiling of tumors, exposing a complicated landscape of genetic abnormalities, epigenetic alterations, and gene expression variations that lead to medication resistance (69). Single-cell sequencing tools contribute to this knowledge by showing the heterogeneity of treatment resistance inside a tumor, detecting discrete groups of resistant cells, and determining their unique genetic properties (70). These developments allow scientists to dissect the complex networks of signaling and molecular connections that cause drug resistance, paving the way for the discovery of novel targets for therapy and markers (71).

Moreover, CRISPR-Cas9 gene editing technology has become known as an effective tool for investigating drug resistance pathways. Scientists may use CRISPR to carefully introduce particular mutations into cancer cells or knock out genes of interest, allowing them to directly examine the effect of these genetic alterations on treatment sensitivity (72). The combination of CRISPR with additional developed molecular methods, like proteomics and metabolomics, offers systems-level knowledge of drug resistance, showing how tumor cells change and adapt under selective pressure (73). This method may be utilized to model drug resistance in vitro, detect new resistance genes, and confirm potential targets for therapy (74).

Emerging therapies to combat drug resistance

The fight against drug resistance in cancer drives therapeutic development. Many interesting treatments are developing; including the invention of next-generation inhibitors that target resistant mutations, such as the application of third-generation EGFR inhibitors (e.g., osimertinib) for lung cancer patients with the T790M resistance mutation (44). Combination treatments are also gaining popularity, attempting to simultaneously address various resistance pathways or to avoid the establishment of resistance. For instance, the combination of a BRAF inhibitor with a MEK inhibitor in melanoma targets two important components of the MAPK pathway, lowering the risk of resistance development (75). Immunotherapy, which utilizes the body's immune system to attack cancer, provides another path for overcoming resistance, especially in tumors with high mutational loads (76). Strategies to modify the tumor microenvironment, including targeting cancer-associated fibroblasts or reprogramming immune system cells, are also being researched to disturb the defensive niche that supports treatment resistance (77). Lastly, personalized medicine techniques, informed by genetic profiling of individual malignancies, are becoming more crucial for identifying the most successful medicines and predicting future resistance mechanisms. Some of these cases are mentioned in the next section (78).

Effective therapeutic combinations for overcoming drug resistance in cancer

The emergence of drug resistance remains an important hurdle in cancer treatment, frequently causing the progression of the disease and treatment disappointment. A promising approach for tackling this obstacle is the application of combination treatments, which involve using two or more drugs concurrently to target various routes or mechanisms inside tumor cells (79). Combination treatment works by targeting cancer cells from numerous sides, making it harder for them to build resistance to all of the medications at once. Ideally, medications in combination ought to have synergistic impacts, which means that their combined impact exceeds the sum of their impacts (80). This synergy may occur via a variety of reasons, including as one treatment sensitizing cancer cells to the other, or medicines targeting complementary pathways required for tumor cell survival. Combination treatment may also assist combat pre-existing resistance processes or inhibit the formation of new resistance mutations (81).

The development of successful combination treatments necessitates an in-depth knowledge of the molecular processes that drive cancer progression and drug resistance. Rational medication combinations frequently depend on the unique genetic changes and signaling networks that are dysregulated in a certain tumor type (82). For example, in tumors with mutations in the RAS-RAF-MEK-ERK pathway, combining a BRAF inhibitor with a MEK inhibitor has been quite successful. BRAF mutations, such as the V600E mutant, persistently stimulate the MAPK system, resulting in excessive proliferation of cells (83). Although BRAF inhibitors might be beneficial initially, resistance frequently arises due to reactivation of the MAPK pathway, which can occur via mutations in MEK or other downstream elements. Combining an MEK inhibitor that targets another stage in the pathway could avoid reactivation while delaying or overcoming resistance (84). Similarly, in tumors with abnormalities in DNA repair routes, combining a PARP inhibitor with a DNA-damaging drug may take advantage of the "synthetic lethality" concept, which states that inhibiting two critical DNA repair pathways results in cancer cell death (85).

Besides directly targeting cancerous cells, combination treatments are being investigated to modify the tumor environment and improve drug delivery. The tumor microenvironment promotes

cancer cell proliferation, survival, and medication resistance. It encompasses many cell types, such as cancer-associated fibroblasts, immunological cells, and endothelial cells, as well as extracellular matrix elements (86). These elements can form an inhibitory microenvironment for tumor cells, shielding them from medications and increasing drug resistance. Combining chemotherapy with medications that target the tumor microenvironment, like angiogenesis inhibitors or fibroblast depleters, may increase drug penetration and therapeutic success (87). In addition, combination therapy may be used to boost anti-tumor immunity and overcome the immunosuppressive environment prevalent in malignancies. Scientists want to harness the immune system's potential to eliminate cancer cells, particularly those resistant to drugs, through the use of immune checkpoint inhibitors with other immunomodulatory medicines or chemotherapy (88).

Checkpoint Inhibitors: Unleashing the Immune System to Overcome Drug Resistance

Immune checkpoint inhibitors are an innovative method of cancer treatment, with the potential to combat medication resistance by reactivating the body's immune system to target and eliminate tumor cells (89). These inhibitors target particular checkpoint proteins including CTLA-4, PD-1, and PD-L1, which usually control immune cell activation and hinder autoimmunity. Cancerous cells often use these checkpoints to elude immune monitoring, essentially placing a brake on immune cell assault. Checkpoint inhibitors function by inhibiting these inhibitory signals, enabling immune cells, notably cytotoxic T lymphocytes (CTLs), to detect and destroy cancer cells (90). This method of action varies greatly from standard chemotherapy or targeted medicines, which often directly target tumor cells and are vulnerable to drug resistance pathways. Since checkpoint inhibitors activate the patient's immune system, the anti-tumor response may persist even after therapy is stopped, possibly resulting in permanent remissions (91).

Checkpoint inhibitors have been effective in a variety of different cancer types, such as melanoma, lung cancer, bladder cancer, and renal cell carcinoma. For instance, ipilimumab, a CTLA-4 inhibitor, has demonstrated considerable activity in melanoma, resulting in increased overall survival for certain patients (92). Likewise, PD-1 inhibitors like nivolumab and pembrolizumab have transformed the management of non-small cell lung cancer, especially in cancers with high PD-L1 expression or a high mutational load (92). These inhibitors have also shown potential in other malignancies, such as Hodgkin lymphoma and MSI-H colorectal cancer. Checkpoint inhibitor effectiveness is often related to the presence of tumor-infiltrating lymphocytes (TILs), suggesting that these medicines need a pre-existing immune response to be effective. Nevertheless, even in tumors with modest TIL levels, checkpoint inhibition may occasionally trigger an immune response, resulting in tumor regression (93).

Furthermore, checkpoint inhibitors may be utilized to combat or avoid medication resistance against additional cancer treatments. For example, in melanoma, combining a BRAF inhibitor with a PD-1 inhibitor demonstrated improved effectiveness in comparison to BRAF inhibitor monotherapy, delaying the formation of resistance to BRAF treatment (94). This shows that inhibiting checkpoints may limit the emergence of resistant clones as a result of targeted therapy's selectivity. In addition, in certain malignancies, checkpoint inhibition has been demonstrated to restore chemotherapy sensitivity in tumors that have become resistant. The methods by which checkpoint inhibitors overcome drug resistance are complicated and poorly understood, but they are most likely a mix of direct immune-mediated death of resistant cells and tumor environment manipulation (95).

Although checkpoint inhibitors have demonstrated exceptional efficacy, they are not without limits. Some individuals react to these treatments, and some may have immune-related side effects (96). The current study aims to uncover biomarkers that forecast responsiveness to checkpoint inhibition, develop techniques to combat resistance to these medicines, and investigate innovative immunotherapeutic approaches like conjunction immunotherapies and adoptive cell therapies. The combination of checkpoint inhibitors with other immunomodulatory drugs, including agonists of stimulatory immunological receptors or inhibitors of immunosuppressive molecules shows potential for improving immunity against tumors and overcoming resistance (97). Continued research and improvement of immunotherapeutic techniques are vital to enhancing results for cancer patients and tackling the ongoing problem of medication resistance.

Nanomedicine: a novel approach to overcoming drug resistance

Nanomedicine offers innovative strategies to combat drug resistance in cancer by improving drug delivery, enhancing drug efficacy, and modulating the tumor microenvironment (98). Nanocarriers, such as liposomes, polymeric nanoparticles, and dendrimers, can encapsulate chemotherapeutic drugs, protecting them from premature degradation and non-specific distribution in the body (56). This targeted delivery minimizes off-target toxicity and allows for higher drug concentrations to reach the tumor site, including drug-resistant cancer cells. Furthermore, nanocarriers can be engineered to actively target cancer cells by attaching ligands, such as antibodies or peptides, that bind specifically to receptors overexpressed on cancer

cell surfaces (99). This active targeting enhances drug uptake by cancer cells, improving the therapeutic index and reducing the exposure of healthy tissues to cytotoxic drugs. Nanomaterials can also be designed to be responsive to specific stimuli within the tumor microenvironment, such as changes in pH, redox potential, or enzyme activity. These stimuli-responsive nanocarriers can release their drug payload specifically at the tumor site, maximizing drug efficacy and minimizing systemic toxicity (100).

Beyond improving drug delivery, nanomedicine can also be used to overcome specific drug resistance mechanisms. For example, multidrug resistance (MDR) often involves the overexpression of efflux pumps, like P-glycoprotein, which actively pumps drugs out of cancer cells (101). Nanocarriers can protect drugs from these efflux pumps, allowing them to bypass this resistance mechanism and reach their intracellular targets. Furthermore, nanocarriers can be designed to deliver multiple drugs simultaneously, including drugs that target different resistance pathways (101). This combination therapy approach can be highly effective in preventing or overcoming drug resistance, as it becomes more difficult for cancer cells to develop resistance to multiple drugs at once. Nanomaterials can also be used to deliver gene therapy agents, such as siRNAs or miRNAs, that can silence genes involved in drug resistance, restoring drug sensitivity in cancer cells (102). For instance, nanoparticle-mediated delivery of siRNA targeting P-glycoprotein has been shown to reverse MDR in various cancer cell lines. The versatility of nanomedicine makes it a promising platform for developing personalized cancer therapies that can be tailored to the specific drug resistance mechanisms present in individual tumors (103).

DISCUSSION AND CONCLUSION

Drug resistance remains a challenging barrier in cancer therapy, with substantial implications for therapeutic effectiveness and patient outcomes. As previously stated, the processes producing drug resistance are diverse and multidimensional, including genetic changes, epigenetic alterations, altered drug metabolism, increased DNA repair, efflux pump function, and interactions with the tumor environment (104). This complexity needs a multifaceted strategy for combating drug resistance, which includes the discovery of new medicines, the optimization of current treatment regimens, and a better understanding of the complicated interaction between cancer cells and their environment (105). Advances in molecular biology, including next-generation sequencing and CRISPR-Cas9 gene editing, have offered crucial tools for unraveling the genetic basis of drug resistance, opening the door for the discovery of novel therapeutic targets and biomarkers (105, 106). These findings have fuelled the development of novel treatment techniques, including next-generation inhibitors, combination therapies, immunotherapies, and nanomedicine approaches, all targeted at combating or avoiding drug resistance (107).

Combination therapy rationally developed based on the unique molecular changes that drive cancer development and resistance, has shown great promise. These combinations may avoid or overcome resistance by addressing numerous routes or processes at the same time (108). Immunotherapy, especially immune checkpoint inhibitors, has transformed cancer treatment by utilizing the immune system's capacity to target and eliminate cancer cells, including those that have gained medication resistance. Nanotechnology provides another novel strategy by improving medication delivery, increasing treatment effectiveness, and altering the tumor environment (109). Nanocarriers may prevent medications from degradation, deliver them directly to cancer cells, and even circumvent efflux pump function, providing fresh hope for overcoming multidrug resistance. The combination of these novel techniques, led by personalized medicine tactics based on genetic profiling of individual tumors, has enormous potential to improve cancer treatment results (110).

Despite tremendous development in the past few years, certain limits persist. It is difficult to predict which patients will acquire medication resistance and to determine the exact resistance systems involved (111). While certain biomarkers have been found, additional study is required to provide more accurate and complete predicting systems. Furthermore, the development of new drugs and therapies is often a lengthy and expensive process (112). Clinical trials are essential for evaluating the safety and efficacy of new treatments, but they can be time-consuming and may not always be successful. Finally, access to these advanced therapies can be limited by cost and availability, creating disparities in cancer care (113).

Looking to the future, several promising avenues of research are being explored. Liquid biopsies, which detect circulating tumor DNA or RNA in blood samples, offer a non-invasive way to monitor treatment response and detect the emergence of drug resistance (114). Artificial intelligence and machine learning are being applied to analyze vast amounts of data from cancer genomics, proteomics, and clinical trials to identify new drug targets and predict treatment outcomes (115). The development of more sophisticated Nanocarriers, capable of delivering multiple drugs or gene therapy agents simultaneously, holds great promise for overcoming complex drug resistance mechanisms (116). Continued research into the tumor microenvironment and its role in drug resistance will pave the way for new therapeutic strategies that target

not only cancer cells but also the supporting cells and the microenvironment itself. By addressing the current limitations and pursuing these future directions, we can move closer to a future where drug resistance is no longer a major obstacle to successful cancer treatment.

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Author contributions

Yousef Roosa and Neda Abedi were involved in the conceptualization, design and writing of the manuscript draft. The authors read and confirmed the final manuscript.

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Therapeutic Candidates for COVID-19: A Comprehensive Review of Antiviral, Immunomodulatory, and Emerging Treatments

Hossein Fazli[1,*]

[1]Assistant professor of anesthesiology, school of Medicine, Alborz University of Medical Science, Karaj, Iran.

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Corresponding author: Hossein Fazli, Assistant professor of anesthesiology, school of Medicine, Alborz University of Medical Science, Karaj, Iran. Email: drhf_md@yahoo.com

ABSTRACT

Background and Objectives: The coronavirus first appeared in Wuhan, China, in late November 2019, and has since spread to more than one hundred countries. COVID-19 has been declared by the World Health Organization as a Public Health Emergency of International Interest. This has been the result of a virus now having reached pandemic proportions and there not being an effective vaccine or antiviral treatment. In this article, we aim to highlight each current drug being tested for potential effectiveness on this disease.
Methodology: The research is a descriptive review conducted by a search in reputable scientific databases, including Scopus, Google Scholar, and PubMed, utilizing the phrases virus, coronavirus, COVID-19, SARS-CoV-2, and treatment. The latest expertise:given that the development and efficacy of antiviral drugs require substantial time, monotherapy for other diseases may represent the most efficient therapeutic option for a certain condition. Pharmaceuticals with broad-spectrum efficacy, including Bevacizumab, Methylprednisolone, Fingolimod, fluoxetine, Ritonavir, chloroquine Fesnate, remdesivir, and Favipiravir, are currently under investigation as prospective candidates in various clinical trials.
Conclusion: To conclude, all these drugs are potentially useful in the prevention and treatment of diseases. But none of these drugs is a cure-all, specific treatment for COVID-19. Therefore, we must continue to search for an effective drug treatment for this disease until we have a proven successful agent available. May you own for all of eternity.

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INTRODUCTION

COVID-19, caused by the SARS–coronavirus 2 (SARS-CoV-2), was first identified in late November 2019 in Wuhan, China, and has since developed into a global health emergency (1, 2). COVID-19 is declared a Public Health Emergency of international concern by the WHO With the epidemic growing into a pandemic, and no antiviral drugs or vaccinations available yet, new therapeutics strategies need to be developed (3). But after SARS in 2003 and MERS in 2012, a new member of the coronavirus family has given itself to the human population, making the challenge a different one. Like SARS-CoV, SARS-CoV-2 is activated by its interaction with the enzyme ACE (angiotensin-converting enzyme) within the host cell (4). before the lungs, SARS-CoV-2 is found across the whole body nasal tract. ACE-2 is thought to play an important role in protecting humans from lung injury and airway epithelial inflammation, but after viral infection, the protein expression of ACE-2 decreases (5). It is a beta-coronavirus, like SARS and MERS. Sequencing of the SARS-CoV-2 genome showed 96% homology with bat coronaviruses, and 79.6% homology with SARS-CoV. No approved drug or vaccine for COVID-19 is available; however, many clinical trials are underway (6). Clinical trials have used lopinavir and ritonavir.

Delivering new medicine is a long process involving years between discovery research and clinical approval (7). Some recent studies have used viral proteins to screen molecular biomarkers. The results of this work may help in selecting suitable drug candidates in laboratory and clinical settings (8). On the other hand, the use of drugs approved for specific diseases and found to be safe for human use needs to be assessed for a new disease. Such a pharmacological approach is especially beneficial in cases of life-threatening illness if there is a substitute medication or vaccine available (9). However, clinical trials still need to confirm whether the drug is safe for humans. It also is appropriate for this treatment (5). Chloroquine phosphate, previously used to treat malaria, is one of the drugs being explored for this disease's treatment (10). Method: An in silico analysis on 39 drugs performed by the researchers revealed that chloroquine phosphate has anti-COVID-19 activity. Proteasome inhibitors, including lopinavir and ritonavir (which are approved for the treatment of other viral infections), are also the subject of clinical trials (11, 12). Despite 96% homology between the M protein of SARS-CoV and SARS-CoV-2, early studies used lopinavir and ritonavir against the M protein of SARS-CoV. Understood that the sequence similarities between a potential COVID19-associated protein and the SARS-CoV M protein could be utilized to model the Mpro-2CoV-SARS-associated protein. It has been recently demonstrated that using homology-based models to screen a library of small molecules for potential COVID-19 therapeutics will be quite advantageous (13).

METHODOLOGY

This study focuses mainly on the treatment prospects for the distinct COVID-19 disease. This study is a prospective one, which has been done by searching the scientific and academic databases PubMed, Google Scholar, and Scopus with keywords virus, coronavirus, COVID-19, SARS-CoV, and treatment as well new information. It was used to obtain the newest information from the world's most respected health agencies, such as the World Health Organization and the Centers for Disease Control and Prevention.

Manifestations of disease

The main symptoms of SARS-CoV-2 infection in humans include fever, fatigue, dry cough, and dyspnea (Table 1). A significant percentage of patients with COVID-19 have deteriorated and remained in critical (14). The main consequences of this phenomenon are acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) and increased the trend of incidence of pneumonia. Another key component of the pathophysiology of ARDS/ALI is supplemental oxygenation, mechanical ventilator, or ECMO (15).

Therapeutic Approaches

One type of process uses limited chemistries and therefore produces a small variety of existing molecules as potential pharmaceuticals. The first line treatment gives antiviral agents with a high attraction to the virus (16). In Europe, interferon and ribavirin, and cyclophilin inhibitors have also been used for the treatment of coronavirus pneumonia (17). The advantage of different types of treatment such as metabolic impact, doses, side effects, and also adverse side effects have been clear (18), and proven in the treatment of viral diseases. However, these drugs have a "very high affinity for steroids," and cannot only target coronaviruses, and their side effects should be considered seriously (19). A second method makes use of existing repositories of molecular data to screen compounds that may be therapeutically interesting for coronaviruses (20). This is possible to screen for these agents, and the effect of these new therapeutic compounds can, therefore, be investigated by this method. For example, the antiretroviral agent lopinavir/ritonavir (21). A third method is to directly use genomic and pathophysiological data from multiple coronaviruses to derive new targeted therapeutics (22). In principle, however, drugs engineered in this manner will have better anti-coronavirus therapies; however, formulation research for new medicines is very likely to take many years, perhaps more than a decade. Table 1 lists several of these medications and their mechanisms of action.

Categories of pharmaceuticals suggested for the treatment of viral infections

Bevacizumab

VEGF is known to be the strongest vascular permeability enhancer. Patients with COVID-19 displayed significantly higher VEGF levels than healthy individuals (23). Hypoxia and inflammation and the deregulation of the respiratory epithelium may lead to increased levels of VEGF. VEGF has been shown to play an important role in mediating vascular permeability and pulmonary edema in the pathogenesis of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), and multiple studies have confirmed this (24). As a result, the anti-VEGF agent bevacizumab could be a potential therapeutic option for this disease. It is an FDA-approved drug for the treatment of ARDS/ALI caused by COVID-19 (25). Bevacizumab is an extended half-life humanized monoclonal antibody. And, it was approved by FDA on February 26, 2004, and is now widely used in clinical cancer it pharmacokinetics and pharmacodynamics from available (26). Consequently, bevacizumab

This medicine appears promising for treating ARDS/ALI and for reducing morbidity and mortality in severely ill patients with COVID-19 by attenuating

Table 1. Mechanism of the effectiveness of different drugs

Drug	DrugMechanism of Effectiveness	Additional Information
Remdesivir	Inhibits RNA-dependent RNA polymerase (RdRp), disrupting viral replication	Used primarily for treating COVID-19; works by incorporating into viral RNA, causing premature termination
Chloroquine/Hydroxychloroquine	Increases endosomal pH, inhibiting viral/cell fusion and glycosylation of cell receptors.	Initially used for malaria; investigated for COVID-19 treatment; potential immunomodulatory effects.
Methylprednisolone	Reduces inflammation by suppressing the immune response.	Corticosteroids used for various inflammatory and autoimmune conditions; can reduce cytokine production.
Combination of Ritonavir and Lopinavir	Ritonavir inhibits the breakdown of lopinavir, enhancing its antiviral activity against HIV.	Used in HIV treatment; ritonavir acts as a pharmacokinetic enhancer.
Favipiravir	Inhibits viral RNA-dependent RNA polymerase, preventing viral replication.	Broad-spectrum antiviral; used for influenza and investigated for COVID-19.
Fingolimod	Modulates sphingosine-1-phosphate receptors, reducing immune cell migration to the central nervous system.	Used for multiple sclerosis; prevents lymphocyte egress from lymph nodes.
Bevacizumab	Inhibits vascular endothelial growth factor (VEGF), reducing tumor blood vessel formation.	Monoclonal antibody used in cancer therapy; targets angiogenesis.

pulmonary edema (27). Phase 2 and 3 clinical trials of this drug were conducted at Shanghai University Qilu Hospital and enrolled 20 patients with severe clinical symptoms of COVID-19. These studies showed that this drug as anti-VEGF treatment can alleviate VEGF induced symptoms (28). “We use methylprednisolone, which is a type of cortisone. Methylprednisolone is used to treat a variety of inflammatory conditions including rheumatoid arthritis, lupus, psoriasis, ulcerative colitis, allergy diseases, endocrine diseases, and diseases of the skin, eyes, lungs, stomach, nervous system or blood cells (29). Prednisolone and methylprednisolone may also be used in the treatment of other viral infections, including respiratory viruses such as coronaviruses (27). Two clinical trials have been reported on the research to 80 patients with COVID-19 (24). In another study in Shanghai, 24 COVID-19 patients received the medication intravenously at a dose of 1-2 mg/kg/day for three weeks, whereas in Wuhan Respiratory Hospital, China, it was used in 50 patients. At Hongji Hospital, 40 mg/kg was given intravenously for 7 days (28).

Administration of this medicine depends on the clinical manifestations of the disease in different people, and needs to be done cautiously (30).

Fingolimod: Although immunosuppressive drugs are generally contraindicated for SAR-CoV-2 pneumonia (29), the pathological features of edema and hyaline membrane noted support consideration of appropriate immunosuppressive therapy and ventilatory assistance in patients who are developing ARDS (30). Fingolimod (720FTY) is an immunomodulatory drug commonly used in the management of scleroderma (31). Currently, research is being done to see if fingolimod could help in fighting COVID-19. Results from a Phase 2 clinical trial of this drug involving 30 patients with COVID-19 at the Venice Hospital were reported to provide good clinical outcomes in modulating the immune response of the patients (24).

Chloroquine phosphate

The virus usage and the potential of anti-viral mechanisms in getting rid of more than one subscriber limit area, starting from anti-IL-6, anti-TGF-β, and other processes so that it does not enter 4 once two times and be attractive to other medications have been an advantage (31-33). Here, this drug has recently been identified as an antiviral in the current study. It interrupts SARS-CoV-2 cellular receptor maturation during viral entry and their functionality (33). In addition, in the late stages of SARS-CoV-2 infection in Vero 6E cells, chloroquine not only has direct antiviral action but also modulates the immune system. (31), in the cumulative view, its antiviral action will be strengthened outward. Orally, chloroquine is distributed throughout the body including the lungs. Using the previously established effective concentration of 90% chloroquine in COVID-19-resistant Vero 6E cells (6.9 μM), which is at physiologically active concentrations (as in the case of plasma of rheumatoid arthritis patients receiving 500 mg (34) as the baseline. It is a 70-year-old drug that is therefore considered safe to use in COVID-19 (34). It has now been removed from worldwide clinical trials because of its negative effects. In Shanghai, the drug was given to 30 pneumonia patients as part of a phase 3 experiment. Another trial has used chloroquine as a prophylactic in 1,000 people (32). These studies showed that this drug reduced the severity of pneumonia, the frequency of symptoms, and the risk of

viral pneumonia without side effects (32).

Lopinavir plus ritonavir

A combination of two drugs used for the treatment of people with drugs under the brand name Kaletra, and is also in trials in Thailand with the flu medicine oseltamivir (Tamiflu) in a regimen for COVID-19 (35). Since these pharmaceuticals block viral proteasomes, they have limited adverse effects (36). A case report followed on 18 February 2020 claiming that an old Chinese man, who was the first patient to receive "Thailand cocktail" at a hospital in Bangkok, had completely recovered from acute pneumonia caused by the COVID-19 virus (37). However, this was tested in the 2003 SARS pandemic, and the results found that patients with first-line therapy of lopinavir/ritonavir fared better, with one patient showing a decreased viral load (38). One patient given methylprednisolone developed a respiratory tract infection (39). Therefore, and indirectly, through reducing the damage done by the immune system, both antivirals may represent a suitable dual treatment for the new coronavirus (40). In this context, a phase 4 clinical trial of this drug was performed with 400 COVID-19 patients at Tongji Hospital. A study was done in the Wuhan Jinyintu Hospital with 125 patients (24). At Wuhan Jinyintu Hospital, the dosing for 80 patients was 200 mg lopinavir and 50 mg ritonavir bi-daily. Results showed that the drug reduced the titer of the virus and improved the patients clinical outcomes (28).

Remdesivir

In culture cells and in mouse and nonhuman monkey models, remdesivir has shown an antiviral effect against RNA viruses, including 5CoV-MERS/SARS (33). Remdesivir is an in vivo viral RNA replication disruptor and is the ribonucleic acid-dependent ribonucleic acid polymerase (RdRp) inhibitor of the ebolavirus (EBV) (34). The Mabulavirus (EBV) phase is active for remdesivir according to analysis. The compound was shown to be effective against EBV infection in vivo. Remdesivir acts as a nucleotide analogue (35). Warren et al. It was shown that intravenous administration of remdesivir at a dose of 10 billion mg per kg achieved blood levels (10 μM) of the active form of the drug and conferred complete protection against SARS-CoV-2 in a non-human primate model (36). The EC50 (olive oil + simonosa glycyrrhizin) of remdesivir against COVID-19 in Vero E6 cells is 1.76 μM, suggesting this is relevant to NHP (37). The EC50 of remdesivir in the SARS-CoV-2-infected Vero E6 cells was 0.77 μM, with a selection index (SI) of 12948. Remdesivir also restricts viral infection in a human cell line (human lung cancer-7Huh cells) susceptible to COVID-19 (36).

Remdesivir is now being evaluated in China in a Phase III, double-blind, randomized, controlled clinical trial. Clinical phase 2 and 3 clinical trials are performing in patient COVID-19 in University of Nebraska and University of Chicago (38). In Phase 3 clinical studies, 452 patients with acute respiratory symptoms or mild to moderate COVID-19 have been given remdesivir as a treatment (24).

Fovavivir

Fovavivir (T-705); 3-6-fluoropyrazinecarboxamide-2-hydroxy (3-hydroxypyrazine) is an antiviral compound with specificity for RNA-dependent RNA polymerases (RdRps) of RNA viruses (39).

Favipiravir undergoes intracellular phosphorylation to become the active form of the drug. RTP-Favipiravir, an RdRp substrate (46), competes with the phosphenolphosphate end of GTP, blocking RNA polymerase activity. Because favipiravir shares an conserved catalytic domain of RdRp with multiple RNA viruses, its broad-range antiviral activity may be an advantage. Favipiravir is effective against Influenza viruses, including those resistant to antiviral drugs. The active ingredient favipiravir has proven antiviral efficacy against a variety of highly pathogenic RNA viruses, including arenaviruses, bunyaviruses, and filoviruses that cause severe hemorrhagic fever (40). The drug was used in bovine studies during the SARS-CoV-2 pandemic of 2019 (41). In a controlled trial by Chun et al. Participants in (2020) had moderate symptoms, recovered within 7 months of treatment, and the medicine reduced the incidence of severe acute respiratory syndrome (SARS-CoV-2)(42). A study in a Swiss hospital of 80 patients treated with favipiravir also showed it was better than lopinavir-ritonavir (43).

DISCUSSION

Antiviral Drug Development 7 Years Ahead of Its Time. This rapid advancement is fueled by the need for effective treatments for pandemics and other widespread threats to health (44). Many of them are actually old medicines, originally designed to treat other problems, but are now indispensable for the new problems. At present other antiviral agents are being investigated in clinical trials as potential therapy for COVID-19 (45). They include bovisumab, prednisolone methionine, fingolimod, fluoxetine, ritonavir, chloroquine phosphate, remdesivir, and favipiravir. Each of these medications has a unique mechanism of action, providing a multi-faceted approach to fighting the virus. They are critical to evaluating their efficacy, appropriate dosages, and safety for treatment of COVID-19(46).

Though we have potent treatments available to combat most forms of viral infection, the ongoing SARS-CoV-2 pandemic has highlighted that our therapeutic options for dealing with mononucleosis-inducing coronavirus infections are extremely limited

(47). The SARS outbreak in 2003 and the 2012 MERS-CoV outbreak reduced considerable research initiative, however as of now no pharmacological treatment for mononucleosis-inducing coronaviruses exists(48). The severity of this disease is a key reason why no primary coronavirus has been successfully isolated so far. While SARS-CoV-2 falls under scrutiny, new viruses might threaten in the future (49). They are a global public health problem. Thus, how to modulate the virulence of human coronaviruses is an affirmed scientific task (50). Because of limited resources for antiviral drug development and commercialization, existing therapeutics for other conditions may provide the only and fastest alternative of therapy for emerging infectious diseases. The majority of the medications in question have substantial experience, with efficacy well studied and safety well established (51).

First, they bind to the RdRp of SARS-CoV-2, thereby interfering with viral RNA production. That work indicated that TP-Remdesivir and Favipiravir bind strongly to the RdRp of SARS-CoV-2, consistent with their core antiviral mechanisms, indicating that these two drugs may be suitable for treating SARS-CoV-2-induced pneumonia (52). According to the analytical results, this study discovered for the first time that Remdesivir binds to one of human 2TMPRSS, an important protein known to mediate viral replication, which provides possibilities for further investigation (53). Subsequent investigations suggest that chloroquine phosphate has greater anti-SARS-CoV activity. This medicine is unique yet unproven. Research in bioinformatics suggest that chloroquine phosphate has interaction with b3Nsp and the E channel. However, more studies are needed to confirm this finding (2). However, this drug has also recently been withdrawn in some countries due to negative impacts. Due to adverse effects associated with the use of lopinavir and ritonavir (these drugs are ineffective and dangerous in patients with coronavirus pneumonia), molecular data were analyzed (54). Molecular data suggest that c3Nsp or channel E is the main target of ritonavir, while c3Nsp has been reported as the main target of lopinavir, although it predominantly targets helicases Nsp3c, Nsp3b, and Nrp3c (55). Some of the targets (i.e., b3Nsp, c3Nsp, and channel E) might be false-positive candidates due to poor model performance for small-molecule proteins (56). Neither lopinavir nor ritonavir affects the binding affinity of the target to important proteins such as CLpro, PLpro, and RdRp(57), as shown by bioinformatics research. While this data indicates that SARS-CoV-2 infection may not be exclusively treated with high doses of lopinavir and ritonavir alone(58).

CONCLUSION

Antiviral drugs are developing much faster than the infectious disease themselves, therefore current drugs for other diseases could potentially provide the best and fastest route to therapy against emerging infectious diseases. Currently, multiple antiviral agents such as bovisumab, prednisolone methionine, fingolimod, fluoxetine, ritonavir, chloroquine phosphate, remdesivir, and favipiravir are being investigated as potential treatment candidates in various clinical trials. Thus, the results of this study suggest that synergistic administration of these two medications may benefit the war against this disease. Before anything else it is worth stressing that these medicines are not a cure, nor a specific treatment for COVID-19, and that a search for a specific cure for this disease has to continue until one is found.

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Consent for publication

Not Applicable

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Association Between Semen Paraoxonase1- Activity Level and L55M Gene Variants with Risk of Male Infertility

Hanie Mortezapour [1] , Abasalt Hosseinzadeh Colaga[1*] , Fatemeh Fallah[1] , Ali Taravati[1] , Seyed Gholam Ali Jorsaraei[2]

[1]Department of Molecular and Cell Biology, Faculty of Science, University of Mazandaran, Babolsar, CP:95447-47416, Mazandaran, Iran.
[2]Department of Anatomy and Embryology, Infertility and Health Reproductive Research Center, Babol University of Medical Sciences, P.O. Box: 4136747176, Babol, Iran.

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Corresponding author:
Abasalt Hosseinzadeh Colagar, Department of Molecular and Cell Biology, Faculty of Science, University of Mazandaran, CP:95447-47416, Mazandaran, Iran.
Email: ahcolagar@umz.ac.ir

ABSTRACT

Today evaluation of polymorphisms of the antioxidant enzyme-encoding genes, which affect the activity of antioxidant enzymes, could be used as risk prediction models for male infertility. This study aims to evaluate the coloration of serum paraoxonase (PON1) activity levels in the semen and its L55M gene variants with the risk of male infertility. In a case-control study, semen samples were collected from 80 healthy controls and 128 infertile men at Fatemeh Al-Zahra IVF and Pastor Laboratory (Babol, Mazandaran, Iran). PON1 activity of semen samples was measured by spectrophotometric methods. Genotyping of all individuals based on *PON*1-L55M loci performed by PCR-RFLP and PCR-sequencing and molecular effects of leucine (L) to methionine (M) substitution were investigated by bioinformatics tools. Results showed a significant difference in genotype frequencies of PON1-L55M polymorphism between patient and control groups, and c.163T>A transition effect on the structure and function of PON1 protein. Also, TA genotype (OR=1.754, 95%CI=0.971 to 3.166, *P*= 0.062) and AA genotype (OR=5.067, 95%CI=1.366 to 18.789, *P*= 0.015) were associated with male infertility. Men with the mutant allele (AA+TA) are exposed to be at risk of male infertility (OR= 1.990, 95%CI= 1.118 to 3.54, P= 0.019). Also, the allelic analysis showed that the A allele was associated with the increased risk of idiopathic male infertility (OR= 1.749, 95%CI= 1.143 to 2.676, P= 0.010). Additionally, PON1 activity was higher in the TT (LL) individuals compared to the TA (LM) and AA (MM) men in both groups (LL> LM> MM). Since the PON1-L55M gene variants are related to PON1 activity levels in the semen and serum paraoxonase is known as an important antioxidant calcium-dependent enzyme, and it could be implicated in male infertility. Based on these findings, the presence of mutant allele (A) and or decreasing semen's PON1 level may be an indicator/ prediction factor for male infertility.

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INTRODUCTION

According to World Health Organization (WHO) guidelines, male infertility as a global health issue, refers to a man's inability to cause pregnancy in a fertile female after 12 months of regular, unprotected sexual intercourse (1). Approximately one in 20 adult men in the reproductive age group suffer from male factor infertility (2). Nearly 25% of these cases of male infertility are unknown and defined as idiopathic male infertility (3). Many factors can impact male infertility including epigenetic, genetic, environmental, and lifestyle-related factors (4). One of the main causes of male infertility is oxidative stress (OS). Reactive oxygen species (ROS) are strongly related to impaired spermatogenesis and male infertility (5, 6). ROS is essential for signal transduction pathways, modulation

of activities of redox-sensitive transcription factors, and regulation of mitochondrial enzyme activities in all living aerobic cells (7). ROS includes free radical agents such as superoxide anions (O_2•), hydrogen peroxide(H_2O_2), peroxyl (ROO•), and hydroxyl (OH•) radicals that are highly reactive (8, 9). ROS in low levels is essential for sperm capacitation, acrosomal reaction, hyperactivation, and fertilization but overproduction of ROS deactivates antioxidants in the seminal plasma and causes OS. Many studies have reported that infertile men have high levels of seminal ROS and low levels of total antioxidant capacity (10). Seminal plasma leukocytes and the mitochondria in the spermatozoa are the primary cellular sources of ROS production (1). According to many studies, high levels of seminal oxidative stress have been associated with sperm dysfunction such as imperfect metabolism, morphology, motility and fertilization due to lipid peroxidation in sperm cellular membranes and sperm DNA fragmentation, eventually leading to cell death (11, 12). ROS causes reproductive problems in men by damaging the balance of sex hormones (13). Detoxification of ROS concentrations is conducted by a well-organized antioxidative system that includes non-enzymatic and enzymatic antioxidants. The enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and paraoxonase (PON). Most of these enzymes are encoded by SOD, glutathione peroxidase (GPX), CAT and PON genes (10, 14). Many studies demonstrated that genetic mutations and genetic polymorphisms in these genes including the single nucleotide polymorphisms (SNPs), can reduce the rate of fertilization (15, 16). A wide range of genetic, epigenetic and lifestyle-related factors can break the balance between the antioxidant defense system and ROS and lead to infertility (17). Serum paraoxonase1 (PON1) is a 43 kDa extracellular HDL-associated antioxidant enzyme that is dependent on Ca^{2+} and prevents LDL and HDL oxidation to protect cells against OS (18, 19). Infertility may be caused by hyperlipidemia; therefore, PON1 prevents the occurrence of this disease by its key role in preventing lipid peroxidation (20, 21). Sperm parameters such as concentration, motility, and morphology can be influenced by paraoxonase levels and its activity in spermatozoa (22). The long arm of chromosome 7 between q21.3 and q22.1 belongs to a multigene cluster that consists of PON1, PON2 and PON3 (23). Liver is the predominant source of PON1 and PON3 expression, which can be detected in the blood. However, PON2 is extensively expressed in numerous tissues such as the testis, brain, kidney and liver but is not secreted into the blood (24). All three PON proteins are localized in spermatozoa; however, only PON1 and PON3 were found in Sertoli and Leydig cells (25, 26). PON1 mRNA has been detected in some tissues such as the kidney. It has been documented that the ability of HDL to prevent both the oxidation of LDL and the interaction between macrophages and endothelium by inactivation of PON1 increases (27, 28). Several researchers have reported that PON1 is present at various stages of spermatogenesis; however, the exact role of PON1 in the male reproductive system is unknown (21, 25). More than seven polymorphisms in the coding region and five in the promoter region have been identified in the PON1 gene. It seems that the substitution of glutamine (Q) by arginine (R) at position 192 (Q192R) and leucine (L) by methionine (M) at position 55 (L55M) of coding region are important functional genetic polymorphisms of PON1 protein (29, 30). Genetic analysis demonstrated that the replacement of leucine by methionine at position 55 influences the paraoxonase and aryl esterase activities and the stability of the protein, which can reduce sperm motility (26, 31). Some reports showed that L55M (as known p.L55M, p.Leu55Met or rs854560, c.163T>A) SNP could be a risk factor in male infertility and influence the stability of the protein (19). The L55M polymorphism shows three phenotypes (LL, LM and MM) in which paraxonase activity in the MM form is lower compared with LL and LM (26). As of this date, little research has been conducted on the PON1-L55M gene polymorphism and male infertility. The cytogenetic location (gene position), preferred name, rs number, and Human Genome Variation Society (HGVS) of PON1-L55M are shown in figure 1A. In this present study, we determined the association of L55M polymorphism with male infertility. Additionally, PONase activities, the level of malondialdehyde (MDA) and total antioxidant capacity (TAC) in infertile men of the Mazandaran population (North of Iran), were assessed.

METHODS AND MATERIALS

Subjects and sample collection

In this case-control study, semen samples were collected from 128 individuals with idiopathic male infertility and 80 fertile men without any history of infertility in their first-degree family, forming a healthy control group from January 2020 to October 2022. All semen samples were collected at Fatemeh Al-Zahra IVF and Pastor Laboratory (Babol, Mazandaran, Iran), and stored at -20°C for further use. The infertile men who were referred to these centers had no history of cryptorchidism, orchitis, infectious disease, diabetes mellitus, drug abuse, obstruction of the vas deferens, varicocele, abnormal profiles of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone, as well as abnormal karyotype Y-chromosome microdeletion. Additionally, the women of infertile men women had no reproductive system problems. Moreover,

there were no signs of coronary heart disease (CHD), atherosclerosis, liver diseases, hypertension, diabetes and cancer in both fertile and subfertile cases, which are diseases affecting paraoxonase. The semen analysis was conducted following the guidelines outlined by the World Health Organization (32). guidelines concerning sperm motility (normal ≥ 25%) concentration (normal ≥ 20× 10^6 spermatozoa/ml), and normal morphology (normal ≥ 14%).

This study was conducted following the principles outlined in the Declaration of Helsinki and received approval from the ethics committees of the University of Mazandaran (#IR.UMZ.REC.1399.033).

DNA extraction and PON1-L55M genotyping

Genomic DNA was extracted from semen samples using the conventional salting-out method described by Mwer et al (33). and then stored at -20°C until use. The PON1-L55M gene polymorphisms were genotyped using a polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP).

One pair of primers was designed using oligo primer analysis software ver. 7.0 and listed as follows: forward primer:

5′-GAAGAGTGATGTATAGCCCCAGTT, reverse primer: 5′-AGTGGGCATGGGTATACAGAAA. The amplification reactions were carried out in a total volume of 25 µl, consisting of 100 ng (2 µl) genomic DNA, 10 pmol (1 µl) of each primer, 2.5 µl of 10X PCR buffer, 0.5 µl of four mixed dNTPs (10 mM, Cinnagen Inc, Iran), 1µl of $MgCl_2$ (50 mM, Cinnagen Inc, Iran), and 0.25 µl of 5U/µl *Taq* DNA polymerase (Cinnagene, Co., Iran). The PCR program used for amplification was as follows: 5 min at 94°C, 32 cycles of 30s at 94°C, 30s at 56°C, 30s at 72°C and finally extension step 5 min at 72°C. PON1 gene rs854560 polymorphism was amplified by PCR and then genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. For the PCR RFLP analysis, PCR products were digested with the *Hin1*II restriction enzyme (Thermo Fisher Scientific, USA). The restriction reactions were carried out in a total volume of 10 µl containing: 5µl PCR product (~100ng); 3µl DNAase free H_2O; 1.5 µl 10X fast digest green buffer and 0.5µl (~3 Units) of the restriction enzymes. These components were incubated for 5min at 37°C. Restriction fragments were separated by 1.5% agarose gel electrophoresis and visualized by UV-transilluminators after staining with 1 µg/ml ethidium bromide (34).

TAC, MDA and PON1 activities

The total antioxidant capacity (TAC) level was measured by its ability to reduce Fe^{3+} to Fe^{2+} using the ferric-reducing ability of plasma (FRAP) method (35). Malondialdehyde (MDA) level was determined by its reaction with thiobarbituric acid (TBA) at 100 °C (36). The procedure for these methods in semen fluid was described by Fallah et al (37).

The PONase activity was assessed spectrophotometrically using paraoxon (Sigma Chem, USA) as a substrate, and the absorbance was recorded at 412 nm due to 4 -4-nitrophenol formation (38). Briefly, the paraoxonase activity was measured at 25°C for 3 min after adding 10 µl of seminal plasma to each well containing 150 µl of Tris-Cl (100 mM, pH 8.5) buffer, including 2 mM $CaCl_2$ and 6 mM paraoxon. All results are expressed in U/ml, defined as 1 nmol of 4- nitrophenol formed per minute. The PON1 enzymatic activity was calculated using the molar extinction coefficient 18 053 M^{-1} cm^{-1}.

Statistical analysis

Statistical analysis of the difference in allele and genotype frequencies between controls and infertility groups was performed using SPSS ver. 26.0 (SPSS, Inc., Chicago, IL, USA) software. After assessing the normality of the constant variables, using the Shapiro-Wilk test, quantitative data were presented as mean± SD for normally distributed data, while qualitative variables were represented as a number or percentage. The Hardy–Weinberg equilibrium (HWE) test was used to estimate genotype frequencies. Results were reported by odds ratios (ORs) and 95% confidence intervals (CI). Both clinical and laboratory data were checked for their correlation with PON1 polymorphism. A p-value less than 0.05 (typically ≤ 0.05) was considered statistically significant.

***In silico* analysis**

L55M is one of the most studied polymorphisms associated with PON1 levels and activity. In this study, several bioinformatics tools were used to investigate the molecular effects of this substitution. To predict the effects of L55M substitution on the structure and function of the PON1 protein, the PolyPhen-2 in silico prediction server (http://genetics.bwh.harvard.edu/pph2/index.shtml) was utilized. The SIFT server is an online tool to predict if an amino acid substitution affects protein function (https://sift.bii.a-star.edu.sg/). The MutPred server, based on SIFT, was developed to classify amino acid substitutions (AAS) as benign or disease-associated (http://mutpred.mutdb.org/).To determine the effect of mutation on protein stability and structure, based on the free energy change value (ΔΔG), the I-Mutant 2.0 online server was employed (https://folding.biofold.org/i-mutant/i-mutant2.0.html). SNP & GO is an online server predicting human disease-related mutations in proteins by determining of Reliability Index (RI) (https://snps-and-go.biocomp.unibo.it/snps-and-go/). To assess the

effect of this polymorphism on m-RNA secondary structure, the RNAsnp database was used. For finding PON1 expression in different tissues and establishing the relationship between rs854560 polymorphism and PON1 expression levels in testis, the GTEx portal was consulted. The Kyte-Doolittle scale was employed to detect protein hydrophobic and hydrophilic tendencies before and after the mutation.

RESULTS

A total of 208 individuals participated in this study, including 128 patients with idiopathic male infertility and 80 fertile men as the control group. The average age of the healthy donors was 35.25±7.19. The case group comprised 128 infertile men with an average age of 37.62±9.24. Seminal factors such as motility, sperm count, morphology and volume in both infertile and fertile groups are summarized in Table 1.

The levels of TAC and MDA (as biochemical parameters) in the seminal fluid were assayed by the FRAP and TBA methods, respectively, with results reported in Table 1. The concentration of MDA in the seminal fluid of infertile men (9.65±5.49) and fertile subjects (7.61±2.53) was evaluated. Statistically, the plasma levels of MDA significantly increased in infertile men compared with fertile subjects ($p<0.001$). In contrast, seminal TAC levels were significantly higher in fertile donors than in men with idiopathic infertility (1949.33± 229.56 vs 1513.43±412.65, $p<0.001$). In general, seminal MDA levels were significantly higher, but seminal TAC levels were considerably lower in men with idiopathic infertility than in fertile individuals (Table 1).

Genotyping results

The amplified 259 bp fragment of PON1-L55M, which flanked the PON1 L55M (c.163T>A) loci, was used for SNP genotyping. After digestion of PCR products by *Hin1 II* and subjecting them to electrophoresis on a 1.5 % agarose gel, gene variants were displayed in Fig. 1B-C. In this figure, an undigested single band of 259 bp was detected in LL homozygotes, digested fragments of 125 and 134 bp were identified in MM genotypes, and three different fragments (134, 125 and 259 bp) were obtained for heterozygous genotypes. The direct DNA sequencing revealed that PCR-RFLP results for the three mentioned SNPs are reliable Fig. 1D.

Distribution of genotypic and allelic frequency

The analysis of c.163T>A polymorphism correlation with sperm abnormality and sperm motility showed that these clinical data of infertile patients were associated with c.163T>A gene polymorphism. Statistical analysis showed that the PON1 genotype distribution for the c.163T>A polymorphism did not deviate from the Hardy-Weinberg equilibrium in the fertile ($\chi2= 3.38$, $p = 0.065$) and infertile ($\chi2= 3.52$, $p = 0.060$) subjects (all $p>0.05$).

The distribution of genotypic and allelic frequencies of the L55M polymorphism in infertile and fertile men are shown in Table 2. As shown in Table 2, the TA heterozygous genotype (OR=1.754, 95%CI=0.971 to 3.166, *P*= 0.062) and AA genotype (OR=5.067, 95%CI=1.366 to 18.789, *P*= 0.015) were associated with idiopathic male infertility. Also, A allele carriers (AA+TA) were exposed to the risk of male infertility (OR= 1.990, 95%CI= 1.118 to 3.54, P= 0.019). Also, the allelic analysis showed that the A allele of the c.163T>A polymorphism was associated with the increased risk of idiopathic male infertility (OR= 1.749, 95%CI= 1.143 to 2.676, P= 0.010). In general, the investigation of allele and genotype frequencies

Table 1. Sperm parameters and biochemical parameters of fertile and infertile men.

Drug	DrugMechanism of Effectiveness	Additional Information
Remdesivir	Inhibits RNA-dependent RNA polymerase (RdRp), disrupting viral replication	Used primarily for treating COVID-19; works by incorporating into viral RNA, causing premature termination
Chloroquine/Hydroxychloroquine	Increases endosomal pH, inhibiting viral/cell fusion and glycosylation of cell receptors.	Initially used for malaria; investigated for COVID-19 treatment; potential immunomodulatory effects.
Methylprednisolone	Reduces inflammation by suppressing the immune response.	Corticosteroids used for various inflammatory and autoimmune conditions; can reduce cytokine production.
Combination of Ritonavir and Lopinavir	Ritonavir inhibits the breakdown of lopinavir, enhancing its antiviral activity against HIV.	Used in HIV treatment; ritonavir acts as a pharmacokinetic enhancer.
Favipiravir	Inhibits viral RNA-dependent RNA polymerase, preventing viral replication.	Broad-spectrum antiviral; used for influenza and investigated for COVID-19.
Fingolimod	Modulates sphingosine-1-phosphate receptors, reducing immune cell migration to the central nervous system.	Used for multiple sclerosis; prevents lymphocyte egress from lymph nodes.
Bevacizumab	Inhibits vascular endothelial growth factor (VEGF), reducing tumor blood vessel formation.	Monoclonal antibody used in cancer therapy; targets angiogenesis.

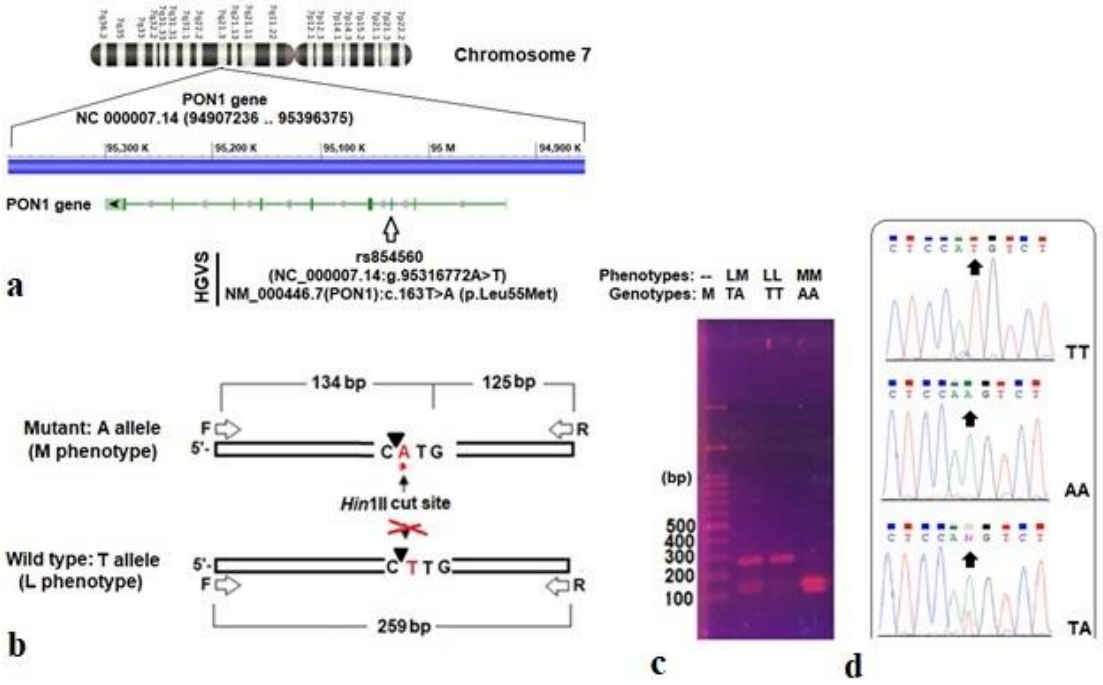

Fig 1. PON1 gene map, RFLP map and DNA sequencing results of PON1-L55M (c.163T>A) loci. a Human PON1 gene map retrieved from the NCBI database, L55M polymorphism located in exon 7. b Schematic of RFLP map, PCR product sizes, and Hin1 II restriction map. c Restriction digest pattern on the 1.5% agarose gel electrophorese, which was stained by ethidium bromide. d Results of PCR-directed sequencing which showed TT, TA, and AA genotypes. M= 100 bp DNA Marker (Fermentas Co., Germany).

Table 2. Analysis of PON1 L55M (c.163T>A) gene variants with the risk of male infertility.

Genotype/ Allele	No. and Percentage		OR (95% CI)	p-value
	Control (80)	Case (n=128)		
Genotype (Phenotype)				
TT (LL)	38 (47.50%)	40 (31.25%)	-	-
TA (LM)	39 (48.75%)	72 (56.25%)	1.754(0.971-3.166)	**0.062***
AA (MM)	3 (3.75%)	16 (12.5%)	5.067(1.366-18.789)	**0.015***
AA(MM) + TA (LM)	42 (54.54%)	88 (68.75%)	1.990(1.118-3.542)	**0.019***
Allele				
T	115(71.87%)	152(59.37)	-	-
A	45(28.22 %)	104(40.62)	1.749(1.143-2.676)	**0.010***

OR = odds ratio; CI = confidence interval; **P value < 0.05***

for the PON1-L55M polymorphism showed that the frequency of the 163A allele in the infertile was higher than the control group (40.62% and 28.22%, respectively). Finally, subjects who receive the A allele have an increased risk of idiopathic male infertility.

Analysis of paraoxonase activity in seminal plasma

Our findings revealed a significant difference in plasma PON activity between the infertile and control groups ($p<0.001$). Infertile patients had considerably lower PON activity (0.046±0.002) in semen plasma compared with healthy men (0.063±0.002) (Fig. 2A). Collectively, PON activities in the LL genotype were the highest followed by LM and MM genotypes, respectively (MM< LM< LL) in both patients and controls. (Fig. 2B).

***In silico* results**

According to the PolyPhen-2 online tool, L55M was classified as benign. The L55M polymorphism, submitted to PolyPhen, was also assessed by the SIFT server, revealing a strong correlation between the results obtained from the PolyPhen and SIFT server. There was a notable relationship between the results obtained from PolyPhen and the MutPred servers. As per the I-Mutant 2.0 online server, a negative ΔΔG value suggests that the mutated protein has lower stability, supporting our findings that the L55M substitution can reduce the protein stability (ΔΔG=-0.64). The SNP& GO server indicated that rs 854560 with a Reliability Index value greater than 5 (RI=7) may have disease-causing potential while the cell continues to survive despite it. Each amino acids have numeric values

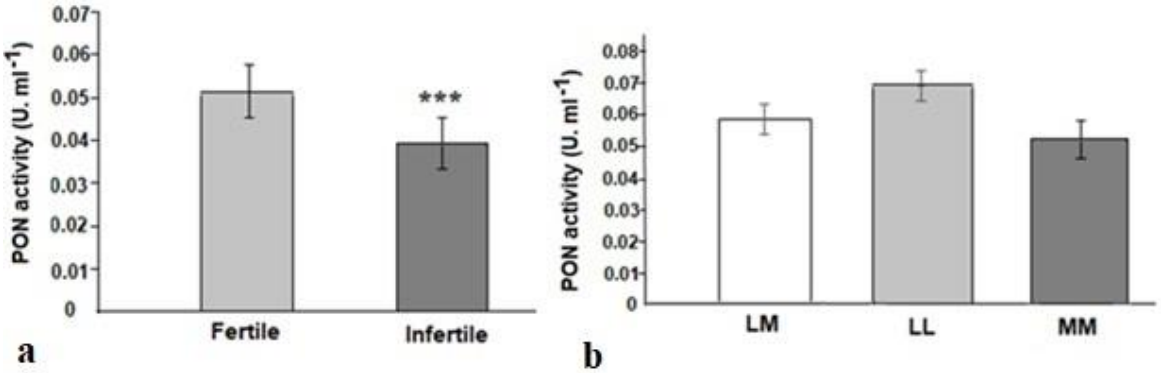

Fig 2. Paraoxonase activity level in the different groups. a PON activity in the infertile and fertile groups, which is a significant difference between the PON activity levels between infertile and fertile groups (***p < 0.001). b Paraoxonase activity in different genotypes, in which PON levels were the highest in the LL genotype followed by LM and then MM genotype (MM<LM<LL) in both infertile and fertile groups.

indicating its hydrophobic or hydrophilic nature, and a change in an amino acid can affect protein hydrophilicity and hydrophobicity. The Kyte scale analysis of PON1 (Kyte-Doolittle Hydropathy Plot) revealed that the peak of hydrophobic pattern in the wild type at position 55 for leucine is -0.44 (Fig. 3B) higher than that for methionine in the mutant variant (-0.82) (Fig. 3A). Consequently, the leucine-to-methionine substitution can decrease the tendency of PON1 protein hydrophobicity (Fig. 3). Using GTEx, PON1 gene expression in different tissues such as adrenal glands, breast, fallopian Tube, liver, nerve, ovary, prostate, testis, thyroid and vagina is illustrated in Fig. 4. According to GTEx portal outcomes, the rs854560 variant is remarkably related to different levels of PON1 expression in the liver, testis and ovary (Fig. 4). The data in the RNAsnp database revealed that leucine substitution by methionine in this polymorphism didn't bring about a significant change in m-RNA secondary structure p = 0.8427; the p-value < 0.2 is a fundamental structural change, (Fig. 5).

DISCUSSION

Infertility is one of the most significant social problems, known as a multifactorial disease. Epigenetic modifications, genetic variants and environmental factors play a key role in the risk of male infertility (2, 39). The male reproductive system may be affected by environmental factors and pollution. Cigarette smoking is one of the main risk factors for male infertility. Acrosome reaction and capacitation, two necessary processes for fertilization, can be impaired by smoking, and smoking can cause sperm ultrastructural abnormalities. According to the American Society of Reproductive Medicine, research shows that cigarette smoking is associated with poorer semen parameters in smokers than in non-smokers (40). Sperm creatine kinase activity can be reduced by using cigarettes (Smoking can lower sperm creatine kinase activity). Cigarette smoking modifies the PON1 thiol groups and then inhibits the hydrolytic activity of this enzyme (41). Pregnant women exposed to smoking during the first trimester of pregnancy showed reduced PON1 activity (42).

Stress oxidative (OS) and reactive oxygen species (ROS) are strongly related to impaired spermatogenesis and male infertility (5). ROS has both favorable and disadvantageous effects on spermatozoa, as low levels of ROS are necessary for sperm fertilization, motility, capacitation and acrosome reaction. On the other hand, high levels of ROS may cause male infertility by inducing sperm morphology, membrane and DNA damage (11, 12). The human body has several antioxidant enzymes to protect itself against ROS damage (10, 43). Antioxidant enzymes play a key role in maintaining the oxidant and antioxidant balance (22). PON1 is an antioxidant calcium-dependent enzyme that appears to play an essential role in the development of a large variety of diseases. This enzyme possesses three enzymatic activities: lactonase, aryl esterase, and paraoxonase activity. PON1 is mainly synthesized in the liver, then transported from the liver to several tissues and released into the blood circulation where it binds to cell membranes and protects lipids against peroxidation (44). PON1 received its name from its ability to hydrolyze paraoxon (diethyl p-nitrophenyl phosphate). Several studies have reported that PON1 is present at various stages of spermatogenesis (21, 25). The sperm plasma membrane has a high level of polyunsaturated fatty acids. The PON1 gene encodes an enzyme that protects the sperm plasma membrane against lipid peroxidation (22). There is enough evidence showing that genetic risk factors play an important role in increasing the risk of male infertility (45, 46). L55M is one of the most common types of genetic variation in the coding region identified in the PON1 gene, and it affects PON1 serum concentration

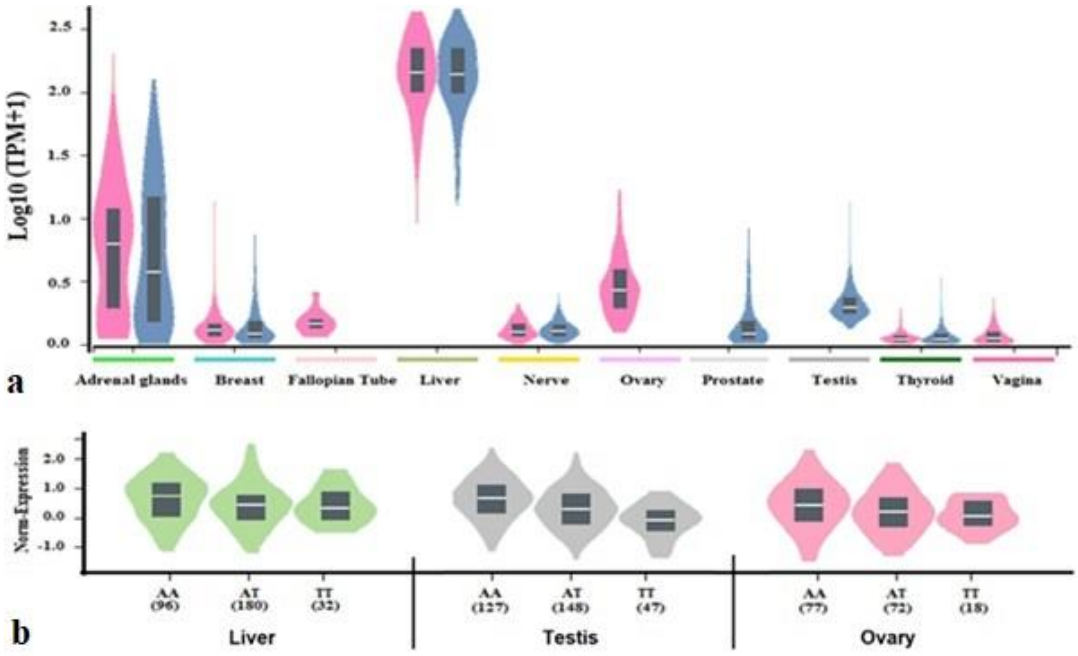

Fig 4. The data deduced from the GTEx server showed. a The expression of PON1 in different tissues such as adrenal glands, breast, fallopian tube, liver, nerve, ovary, prostate, testis, thyroid and vagina in female (pink) and male (blue). b Result of the GTEx portal revealed that leucine substitution by methionine is appreciably related to different levels of PON1 expression in the liver, testis, and ovary.

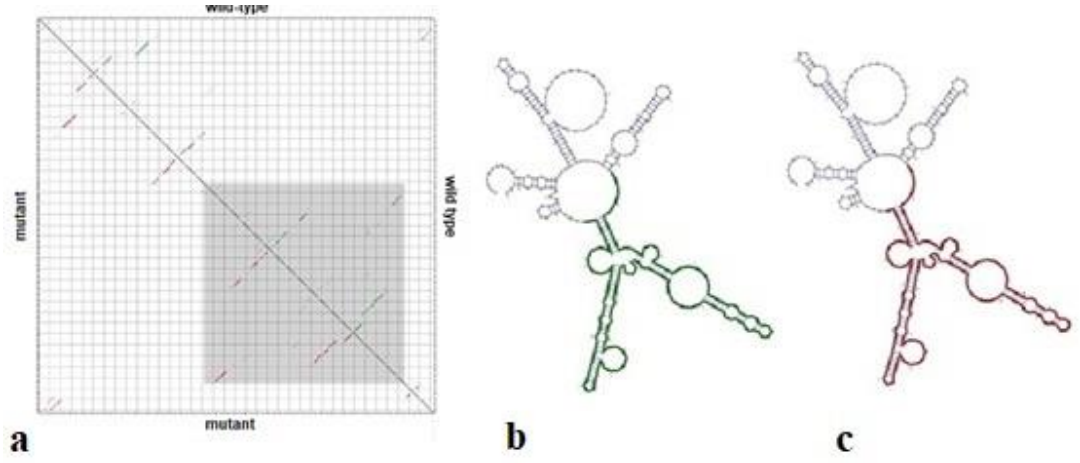

Fig5. The effect of L55M polymorphism on PON1 mRNA secondary structure. An RNA structure alignment plot for the mutant and normal variety. b The wild type secondary structure is depicted in green vs. c The mutant variant secondary structure is depicted in red.

and activity (47). PON1 L55M polymorphism has been reported in various diseases, including cardiovascular diseases (48), rheumatoid arthritis (49), Breast cancer and Parkinson's (28). In this present study, the TA heterozygous genotype (OR=1.754, 95%CI=0.971 to 3.166, *P*= 0.062) and AA genotype (OR=5.067, 95%CI=1.366 to 18.789, *P*= 0.015) were associated with idiopathic male infertility. Also, A allele carriers (AA+TA) were exposed to the risk of male infertility (OR= 1.990, 95%CI= 1.118 to 3.54, P= 0.019). Also, the allelic analysis showed that the A allele of the c.163T>A polymorphism was associated with the increased risk of idiopathic male infertility (OR= 1.749, 95%CI= 1.143 to 2.676, P= 0.010). We found a significant association between the substitution of leucine (TTG) at position 55 by methionine (ATG) at the third exon (L55M) and the risk of male infertility. Previous studies show conflicting results. For example, in 2018, Behrouzi et al investigated the association between PON1 192 Q/R polymorphism and the risk of idiopathic male infertility in Northern Iran. Their result indicated that the PON1 192 Q/R polymorphism is associated with a decreased risk of idiopathic male infertility. In 2020, Alizadeh et al investigated the association between PON1 L55M and Q192R polymorphisms and recurrent pregnancy

loss risk. In their study, statistical analysis of the L55M polymorphism for the MM genotype in the case group compared with the control group showed a significant difference, but none for the LM and LL genotypes (50). The contradictory results of different studies can be caused by the differences in sample sizes, and geographical, racial, and environmental factors.

The L55M polymorphism (rs854560, c.163T>A) displays three phenotypes (LL, LM and MM) in which paraxonase phosphodiesterase activity in the MM form is lower than LL and LM (26). Although serum PON1 aryl esterase activity wasn't linked to this polymorphism, L55M can regulate PON1 aryl esterase activity in the blood (42). M allele carriers have significantly lower PON1 mRNA and protein levels. Some research has suggested that men with MM genotype might be predisposed to infertility. On the other hand, it was shown that the c.163T>A genotype is associated with less sperm motility. As a keynote, L55M could be a risk factor in male infertility and influence the stability of the PON1 protein (19).

In Conclusion, the present study establishes an association; L55M polymorphism can be a genetic marker for male infertility in the Iranian population. One limitation of this study was the sample size. We suggested that future studies focused on some factors such as environmental and epigenetic factors in a larger sample size to the significance of these findings.

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Authorship Contribution Statement

All authors contributed to the writing of this article: **AHC** was involved in the conceptualization, methodology, and software sections. **HM** and **FF** contributed to the material preparation, data curation and analysis, and preparation of the original draft. **AT** and **GJ** contributed to the data analysis and editing of the manuscript.

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Availability of Data and Materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

Informed consent was obtained from all subjects, and the study protocol was approved by the ethics committee of the University of Mazandaran and conducted in accordance with Iran National Committee for Ethics in Biomedical Researches (#IR.UMZ.REC.1399.033).

Declarations

Consent to Participate

As corresponding author, I confirm that the manuscript has been read and approved for submission by all the named authors.

Consent to Publish

All authors would like to submit this manuscript, to be considered for publication as a Research Article, in the Personalized Medicine Journal journal. We declare that it is original, has not been published before and is not currently being considered for publication elsewhere.

Conflict of Interest

There is no conflict of interest to declare.

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Approaches to Traditional Vaccines and the Development of New Person-Centered Vaccines

Ramesh Ranjbar [1*], **Romina Hosseinzadeh**[2]

[1]Department of Genetics, Faculty of Basic Sciences, Shahrekord Islamic Azad University, Shahrekord, Iran.
[2]Department of Biology, Faculty of Basic Sciences, East Tehran Branch, Islamic Azad University, Tehran, Iran.

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Corresponding author:
Ramesh Ranjbar, Department of Genetics, Faculty of Basic Sciences, Shahrekord Islamic Azad University, Shahrekord, Iran.
Email: Ranjbar.Ramesh@gmail.com.

ABSTRACT

According to the World Health Organization, immunizations save between two and three million lives every year by avoiding illness. In addition to these immunizations, eradicating human smallpox was possible and is close to eradicating polio. In addition, vaccines have a significant economic impact because they prevent hospitalization of patients and other care costs. A vaccine is a biological product that specifically leads to acquired immunity against a pathogenic pathogen and prevents the disease in the face of the main pathogen in a person. Therefore, vaccines are an important tool for maintaining health in the global community. Traditional vaccines have been used against a wide range of pathogenic pathogens, both viral and bacterial, and have been successful. However, these vaccines do not work and are ineffective against pathogens that change rapidly in terms of genetic material and surface epitopes.

During the last decade, vaccines based on nucleic acids, viral vectors and biomaterials have shown promising results. This study has discussed an overview of traditional vaccines, mRNA-based vaccines, viral vector-based vaccines, and biomaterials.

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INTRODUCTION

According to the World Health Organization, immunizations save between two and three million lives every year by avoiding illness. In addition to these immunizations, eradicating human smallpox was possible and is close to eradicating polio. In addition, vaccines have a significant economic impact because they prevent hospitalization of patients and other care costs (1-3).

Traditional vaccines have been effective against many diseases. Still, there are infectious diseases for which no effective vaccine has been definitively developed, such as human immunodeficiency virus (HIV), tuberculosis (TB), and respiratory syncytial virus. (RSV), cytomegalovirus (CMV), herpes simplex virus (HSV), and Epstein Barr (EBV) (4). In addition, other infectious agents such as the Ebola virus, Zika virus, and acute respiratory syndrome virus have become major threats to global health (3).

Vaccine development began in 1791 by Edward Jenner, who noticed that people who received cowpox had a much milder illness than the original disease (5).

Since then, safer and engineered vaccines have been developed, for example, inactivated/live-attenuated pathogen vaccines (6), subunit vaccines (7), immune epitopes (8), and various classes of adjuvants have significantly increased long-term immunogenicity (9). Newer vaccines produce higher antibody titers and also have fewer side effects (10). After three hundred years since the first vaccination, there are still

many challenges in the development of new vaccines, including low stability, inefficient delivery, and lack of translation in human cells (11, 12).

Fighting the spread of such diseases requires making vaccines with a new method, which is usually not possible with traditional vaccines. These challenges have led to the development of research related to new vaccine manufacturing technologies. Table 1 lists some of the challenges in vaccine development.

The human body has different defense barriers that protect the human body against pathogenic agents. Innate immune systems and acquired immunity are the two components that make up the immune system of an individual. The first barrier of the innate immune system is the skin, mucus and stomach acid, which prevents the entry of pathogenic pathogens (13). After the first barrier of the innate immune system, there are macrophage cells, dendritic cells, monocytes, complement proteins, natural killer cells, mast cells, neutrophils, basophils and eosinophils (14).

The next barrier of the acquired immune system includes B and T lymphocytes. If the innate immune system fails to control the infection, the acquired immune system comes into action. The innate immune system acts non-specifically against all pathogens, but the acquired immune system specifically recognizes and targets the type of pathogen. Also, due to having memory B and T cells, it can create permanent immunity to that pathogen. Therefore, in vaccine production, the goal is to stimulate the acquired immune system to prevent the re-infection of the disease by creating memory cells and appropriate and quick responses و to the pathogen (14).

In general, vaccines work by exposing a person to the whole or part of the pathogen, and as a result, it leads to the activation of the person's immune system.

Traditional vaccines

There are different types of vaccines. Traditional vaccines are live attenuated, killed pathogens, as well as subunit and conjugate vaccines (Fig 1) (15). Live attenuated vaccines consist of a weakened form of a pathogen and can induce a strong immune response. Live attenuated vaccines targeting smallpox, measles, mumps, rubella, and yellow fever are among those that have received clinical approval (16). Although the injection of live weakened pathogens leads to a strong immune response, it can be a risk factor for people with a weak immune system or with underlying diseases. Therefore, an alternative approach such as a completely inactivated pathogen is needed to reduce the risk of disease. Inactivated vaccines are such as hepatitis B virus, poliovirus and rabies vaccines, the development of live attenuated and inactivated vaccines requires the growth of pathogens on a large scale, which is associated with biosafety risk (3).

Subsequently, a pathogen component makes up subunit vaccinations. Subunit vaccines are better in terms of immunogenicity and eliminate the need for pathogen culture. But they often need a booster to create effective immunity (17). The limitations of traditional vaccines have led to the discovery and development of new technologies in the production of vaccines, which include carrier vaccines, nucleic acid-based vaccines, and materials science approaches to vaccination Fig1.

Virus-like Particle Vaccines (VLP vaccine)

One of the unique features of viruses is that viral structural proteins and envelope proteins can self-assemble to form virus-like particles (VLPs) without the viral genetic material. Therefore, this feature can be used to make viral particles without pathogenicity. VIIPs have many applications in medical sciences such as therapy, drug delivery, some diagnostic tests and the development of vaccines (18).

Unlike conventional vaccines, VIPs have many features that make them attractive platforms for vaccine design. They are 20-200 nm in size and also have special geometric structures with multivalent epitopes (19-22) and have the ability to activate helper T cells.

In addition, VLPs are considered harmless because they do not contain the genetic material of the virus and therefore cannot replicate. However, VLPs, like any other vaccine, can cause side effects such as pain and swelling at the injection site. VLP-based vaccines have been developed using viruses that infect humans.

Table 1. Challenges in vaccine development

host variability	Pathogen Variability	Environmental factors
Individual variability	Pathogen diversity	Pollution
Non-responder populations	Hypervariable viruses	Co-infection
Age, Race, Sex, Ethnicity	Antigenic drift	Poor nutrition
	Interactions of Host-pathogen	Obesity
	Immune response evasion	Prior immunity

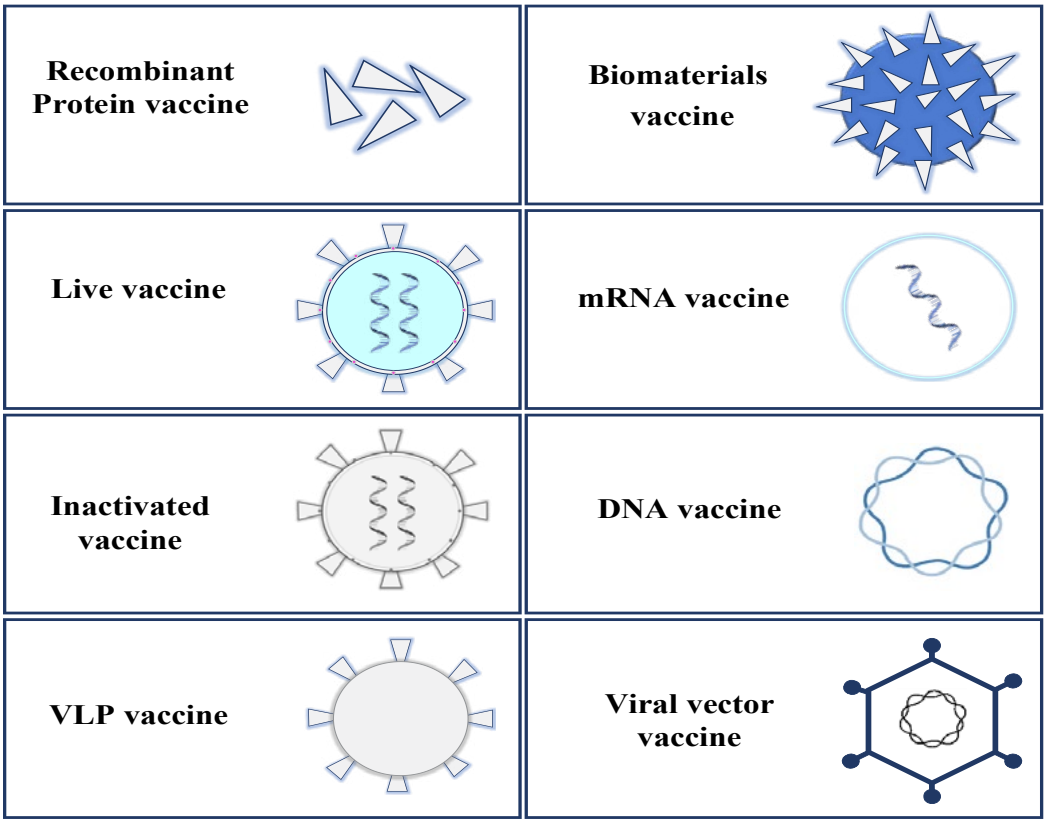

Fig1. All types of vaccines show this figure.

These vaccines are approved against three human viral infections, hepatitis B virus, human papilloma virus and hepatitis E (18).

mRNA vaccine

The concept of vaccines based on DNA and RNA nucleic acids was proposed in the past decades with the hope of being able to develop a flexible, easy-to-produce and safe vaccine. Until the late 2000s, DNA-based vaccines were emphasized due to the stability of RNAs (23). Also, efficient in vivo delivery as well as stimulation of excessive inflammatory responses were obstacles to nucleic acid-based vaccines (Fig1) (24). The production of transcribed mRNA in vitro is a relatively simple process (25-27), but the production of therapeutic, non-infectious and high-quality mRNA that can be well translated and cause serious inflammatory responses has been one of the main limitations in this field. In the early 2010s, the problems facing mRNA by optimizing the coding sequence, and purification of mRNA in the laboratory environment by HPLC to remove possible contaminants in the synthesized mRNA led to the reduction of toxicity and improvement of mRNA performance (28). However, there was still a problem with mRNA stability and efficient cytoplasmic delivery (29, 30). Various approaches have been developed to transfer mRNA into the cell, such as the use of gene guns and electroporation (31). These approaches are complex and expensive, and on the other hand, it is difficult to use them in humans, so the most ideal method is to use a substance that prevents mRNA degradation. In the past few years, many materials have been developed for the efficient delivery of nucleic acids, which have brought significant results (32).

mRNA vaccines work by delivering a fragment of mRNA that corresponds to a protein from a virus or other pathogen. People who receive the mRNA vaccine are not directly exposed to the virus, so they cannot be infected by the vaccine. Using this mRNA, cells can produce viral proteins, and as a natural immune response, the immune system identifies pathogen proteins and secretes antibodies against them (33).

The mRNA in these vaccines is not uniform and is rapidly degraded shortly after injection and after the target protein is made, reducing the risk of toxicity and long-term complications. mRNA vaccines enable the precise design of antigenic proteins and on the other hand, by delivering multiple mRNAs to a cell, it enables the production of multi-protein complexes or protein antigens from different pathogens, and thus a single vaccine It can act against several pathogens (34, 35).

Mechanisms that may affect the response of these types of vaccines by B and T lymphocytes include the half-life of antigen availability, the extent of antigen

presentation by MHC Class I/II, the participation of other components of the innate immune system, and the cytokine-induced environment. by the mRNA molecule itself as well as its delivery substance (23).

Viral vector vaccines

Adenovirus vectors were initially used as a promising strategy for gene therapy for gene transfer and gene therapy and basic studies to analyze gene function (36). Because these vectors have high transfer efficiency, and relatively large capacity (37), and on the other hand, they can infect a wide range of cells, including liver cells, myoblasts, epithelial and endothelial cells, and also induce a moderate level of innate immunity, have high thermal stability (37) Fig1. Therefore, adenovirus vectors are a suitable option for making vaccines. These types of vaccines can be effective in preventing infectious diseases such as the Ebola virus and HIV.

Adenoviruses are non-enveloped viruses that contain linear double-stranded DNA enclosed in a protein capsid.

This group of viruses cause 70 types of diseases in humans and is classified into 7 types (A-G) (38). Infection in humans causes symptoms of cold, sore throat, diarrhoea and vomiting.

Viral vector vaccines use a harmless virus to transfer a piece of genetic sequence to our cells, allowing them to produce pathogenic proteins. The harmless virus acts as a vector to transfer the genetic sequence. Our cells then make the viral protein that was transferred to our cells by the vector and present it to the immune system (39).

However, viral transmission itself plays an important role by enhancing the immune response. Which leads to a more severe reaction to the presentation of the pathogen's genetic sequence to the cell.

Biomaterials vaccine

However three centuries following the initial vaccination, there are still a lot of obstacles to overcome in the creation of novel vaccines, such as poor strength, ineffective administration, and a failure to penetrate human cells (40). To increase the effectiveness, long-term safety, and durability of vaccines, biomaterials including lipids, microneedles, scaffolds, and other material transporters, as well as natural and artificial polymers, have been created within the last three decades(Fig1) (41, 42).

Biomaterials offer a unique strategy for safe cargo delivery, protection, modification and management for targeted delivery, minimizing the number of injections and reducing systemic toxicity (43-45).

Biocompatibility, adjustable immunity, minimal inflammatory reactions, and comparatively high stability across various vaccine administration classes are just a few of the numerous benefits that biomaterials provide. Several types of biomaterials have been developed in micron and nano sizes, there are a large number of biomaterials, few of which offer sustained release properties (40).

Synthetic biodegradable polymers, including polylactic acid (PLA), polylactic-glycolic acid (PLGA), polyurethane (PU), and poly ε-caprolactone (PCL), are the most widely used biodegradable polymers in medicine (46-48).

Meanwhile, PLGA copolymers have been recognized as safe by the FDA and are suitable as a carrier for the sustained release of antigens and vaccine adjuvants due to their safety profile (49, 50).

Another type of biomaterials is polysaccharides, which are composed of carbohydrate polymers and are made of monosaccharide subunits. Polysaccharides form a very wide category of compounds found in plants, bacteria, fungi and even mammals (40). There are different types of polysaccharides, such as alginate (51, 52), cellulose (53), chitosan (54), hyaluronic acid (55), and starch (56), which have been evaluated in vaccines.

Approaches that combine immunogens with biomaterials have emerged as a promising approach for various types of vaccines (57, 58).

DISCUSSION

In the past decade, significant progress has been made in the field of making new vaccines, including mRNA-based vaccines. These vaccines, by optimizing the design of mRNA and its manufacturing processes, have led to the creation of vaccines that are effective in humans. They can be expressed well and have a higher immunogenicity. Another important feature of mRNA vaccines is targeting multiple pathogens simultaneously. They have also been found in some studies to be able to produce strong and long-lasting responses (57, 58).

Viral vector-based vaccines are also a promising field for developing new vaccines. The design of vaccines based on adenoviruses is based on most of the uncommon and non-pathogenic viruses. The structural components of viruses can be modified and optimized to increase tropism to body cells and tissues to optimally bind to body cells and tissues and express antigens efficiently. Adenovirus-based vaccines can be rapidly developed and produced on a commercial scale. Adenovirus vaccines mentioned suitable features such as stability, no need for cold chain transfer and targeted selection. Also, their use against various pathogens and the flexibility of these vaccines have made them suitable candidates for vaccine production (59, 60).

The use of biomaterials due to their wide spectrum and capabilities such as increased control in the

release of pathogens, targeted delivery, minimizing the number of injections and reducing systemic toxicity has turned them into a promising approach for the production of new vaccines. Also, biomaterials are biocompatible and produce low inflammatory responses and stable immunogenicity. In general, for any pathogenic pathogen, especially diseases such as HIV, TB, RSV, CMV, and Ebola virus that cannot be prevented with traditional vaccines, it is necessary to develop vaccines with a new method. However, it still requires specialized research in the field of each emerging disease (60-63).

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Availability of data and materials

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Consent to publication

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Systematic Review: Application of Artificial Intelligence in Breast Cancer Therapy

Maryam Abbasi Saeidi[1*]

[1]Department of Biology, Faculty of Basic Sciences, Science & Research Branch, Islamic Azad University, Tehran, Iran.

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Corresponding author:
Maryam Abbasi Saeidi, Department of Biology, Faculty of Basic Sciences, Science & Research Branch, Islamic Azad University, Tehran, Iran. Email: maryamabbasisaeidi@gmail.com.

ABSTRACT

Background and Objective: Gene therapy can be employed to treat several disorders, including cancer. Globally, women are more frequently diagnosed with breast cancer than any other cancer type, underscoring the necessity for innovative strategies. Algorithms driven by artificial intelligence can enhance the gene therapy process for breast cancer by analyzing vast data sets, identifying intricate patterns, and classifying those patterns. This project aims to perform a literature evaluation focusing on the therapeutic uses of artificial intelligence in gene therapy for breast cancer.

Materials and Methods: For the aim of this study, data was gathered by reading previously published articles and searching the PubMed database for phrases that were relevant to the question being investigated.

Findings: The AI-driven algorithm analyzes complex molecular pathways in the human body, replicates the knowledge of scientists and physicians in clinical research, and simulates biological processes related to gene regulation, thereby improving the effectiveness of gene vectors, managing gene and drug delivery parameters, and modeling cellular behavior. This method diminishes medical errors and promotes early disease identification and drug efficacy forecasting, thereby providing patients with optimal results from advanced treatments like gene therapy with minimal side effects.

Conclusion: Over the period of the past decade, a multitude of efforts have been made to deploy various gene therapy procedures for breast cancer patients, to achieve the highest possible level of efficacy while minimizing the risk of adverse consequences. As a result, artificial intelligence is considered to be a powerful tool for improving early diagnosis and efficient gene therapy for breast cancer.

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INTRODUCTION

Breast cancer ranks among the most prevalent cancers globally, and progress in its treatment and care is essential for enhancing patient outcomes (1-3). Artificial Intelligence (AI) has demonstrated considerable potential in multiple facets of breast cancer therapy, encompassing diagnosis, treatment optimization, and monitoring. This systematic review seeks to assess the present status of AI applications in breast cancer treatment, emphasizing global practices and developments in Iran. We evaluate the functions of AI in diagnosis, therapy customization, medication development, and monitoring, examining its obstacles, potential, and prospective trajectories (4). The findings demonstrate that AI is revolutionizing breast cancer management worldwide, with notable advancements in Iran; yet, challenges such as technological access, data constraints, and healthcare infrastructure persist (5).

The notion of gene therapy has existed for an extended period, predating the discovery of the first human coding sequence. RNA and DNA are two types of nucleic acids that can be delivered into host cells via

gene therapy. This method is employed to address a variety of ailments (6). Gene therapy is beneficial in treating conditions such as Parkinson's disease and other cancers. Gene therapy is categorized into three types: immunotherapy, oncolytic viral therapy, and gene transfer (7, 8). Breast cancer is the most prevalent disease among women, and due to the inadequacy of current treatments, experts are exploring new therapeutic strategies, including gene therapy. The BRCA1 and BRCA2 genes, essential for DNA repair, are significantly associated with breast cancer (9). Women possessing these genes exhibit a markedly elevated chance of developing breast cancer. The predominant gene therapy methods for breast cancer treatment encompass gene editing, suicide genes, gene silencing, transcription factor targeting via decoy oligodeoxynucleotides, microRNA targeting, aptamer-mediated targeting of breast cancer cells, and DNA or RNA vaccination (10). Given that laboratory processes for identifying an effective and safe gene carrier are both labor-intensive and expensive, it would be advantageous to substitute existing methods with those grounded in artificial intelligence (11, 12). By merging machine learning and molecular biology with the concepts of gene therapy, this expertise can be employed through generative AI to create unique experimental methods for regulating gene expression (13). Artificial intelligence and its associated subfields can accurately simulate biological processes related to gene delivery, assess the efficacy of gene vectors, regulate the parameters of gene and drug administration, model cellular structures, and perform intricate analytical tasks (14). This study aimed to conduct a literature review on artificial intelligence in gene therapy, focusing specifically on its clinical applications for the precise and early identification of genes and gene therapy in breast cancer.

METHODOLOGY

A systematic review of published literature was conducted using databases such as PubMed, Google Scholar, Scopus, and IEEE Xplore. The search terms included "Artificial Intelligence in breast cancer," "AI breast cancer therapy," "machine learning in breast cancer treatment," and "AI in Iran breast cancer therapy." Studies published between 2010 and 2024 were considered for inclusion (Table 1).

Inclusion criteria

Peer-reviewed studies, original research, clinical trials, systematic reviews, and studies that specifically focused on AI applications in breast cancer diagnosis, therapy, or treatment monitoring were included in this study.

Exclusion criteria

Studies not related to breast cancer, studies focusing only on general AI applications with no relevance to therapeutic outcomes and Non-peer-reviewed articles were excluded from this study.

For this article, we examined the abstracts of the publications and selected those we deemed the most relevant. Research is being focused on the promise of nucleic acid-based therapies for both inherited and acquired disorders. Such treatments aim to restore or replace genetic information that has been compromised. In 1991, Rosenberg and associates conducted the inaugural patient-specific gene transfer (15). They administered tumor-infiltrating cells harboring a neomycin resistance gene to five patients with metastatic melanoma utilizing a 3MLV vector (16). Gene expression in cells can be modified by administering nucleic acid-based agents, including DNA, CRISPR/Cas systems, messenger RNA (mRNA), and oligonucleotides. Gene editing facilitates the achievement of this objective (17, 18). Direct gene transfer into cells offers numerous advantages, including reduced immunogenicity; however, it also presents several disadvantages, such as the necessity of employing gene vectors to avert oncogene activation and the inadequate stability of nucleic acids in vivo (19, 20). Gene vectors are categorized into two primary groups: viral and non-viral (21). Virus generation can manifest in various forms, including adenoviruses, retroviruses, herpes simplex virus, and viruses associated with adenoviruses. Increasingly, non-viral vectors are employed for transmission to circumvent issues such as mutagenesis, adverse consequences, restricted gene size, and immunogenicity (22). Polymeric carriers, organic and inorganic nanoparticles, liposomal carriers, and DNA blast injection are non-viral techniques that exclude the use of viruses. Non-viral techniques may possess a distinct physical characteristic (23). Gene therapy has been extensively researched for its potential application in treating various diseases and conditions, including progressive disorders like Parkinson's.

Researchers have investigated gene editing, immune cell engineering, and antibody gene expression as potential gene therapy techniques to eliminate infection-related pathogen receptors (24). A further step in gene therapy is the utilization of viral vectors to develop vaccines for cancer and infectious diseases (25). Gene therapy research encompasses several categories, the most prevalent of which are cancer, genetic disorders (including both single-gene and multigene conditions), infections, and more areas (26).

This chart illustrates the number of publications on AI applications in gene therapy for breast cancer from 2010 to 2024. The bar chart depicts yearly publications, but the line chart demonstrates the cumulative trend

Table 1. The list of studies considered in this study.

Year	Number of Papers	Key Trends/Topics Highlighted
2010	5	Early exploration of AI in genomics
2011	8	Basic machine learning models for gene expression analysis
2012	12	Introduction of gene-editing AI tools
2013	18	AI-aided biomarker discovery
2014	25	Applications in CRISPR and genetic therapy models
2015	30	Growth in AI-driven personalized therapy
2016	45	Integration of AI with imaging and genomics data
2017	55	Advanced AI models for mutation prediction
2018	68	Use of AI for therapy outcome prediction
2019	82	Deep learning in breast cancer gene therapy
2020	95	AI applications during COVID-19 and cancer therapy
2021	110	Accelerated AI applications in precision medicine
2022	130	Large datasets enable more robust AI models
2023	145	Advanced natural language processing in gene therapy research
2024	160 (est.)	Predicted continued growth in research focus

over time.

Gene therapy in cancer

Hematological tumors are the ones that have been the subject of the most comprehensive investigation for gene therapy, followed by cancers that affect the digestive tract and the nervous system (25). It has been established that gene therapy in lung cancer can improve survival rates. This is accomplished through the creation of cancer vaccines, the utilization of viruses to target cancer cells, the induction of apoptosis in cancer cells, and the introduction of genes that either trigger cell death or restore a normal cellular phenotype (27). Breast, pancreatic, liver, and glioma tumors, along with a variety of other malignancies, have all been the subject of additional research studies (28).

The ensuing material explains the three basic categories that represent gene therapy in the field of cancer (29). Immunotherapy, which is intended to improve the immune system's ability to remove cancer cells, has only achieved a limited level of success since cancer cells can avoid being detected by the immune system (29, 30). As a result, the exploitation of gene therapy in the process of developing recombinant cancer vaccines is an innovative approach in the field of immunotherapy (31). On the other hand, in contrast to vaccinations for infectious infections, which are designed to prevent recurrence, these vaccines are meant to cure or control cancer by teaching the immune system of the patient to recognize cancer cells by the presentation of cellular debris that stimulates the immune response (32). In the fields of gene therapy and immunotherapy, CAR T cells are among the most influential and widely recognized devices. It is possible to modify T cells so that they express chimeric receptors on their surface. This process results in CAR T cells, which can react to particular molecules, such as tumor-associated antigen proteins (33). Vectors for oncolytic gene therapy are often viruses that have been genetically manipulated to specifically target and eradicate cancer cells while remaining harmless to healthy cells (34). This has been accomplished through the use of genetic engineering. The vectors, which include vaccinia, adenovirus, herpes simplex virus type I, reovirus, and Newcastle disease virus, have been developed to specifically target cancer cells and cause cell death by viral release, the generation of cytotoxic proteins, and cell lysis (35, 36). A therapeutic strategy known as gene transfer involves inserting a foreign gene into a cancer cell or neighboring tissue (37).

This is done to treat the cancer. For this therapeutic method, several genes have been suggested, including genes that cause suicide, genes that inhibit angiogenesis, and genes that stasis cells (38). For this particular approach, the replication-deficient adenovirus is the primary viral vector that is utilized. Direct DNA transfer, oligodendromeric DNA envelopes, and electroporation are all examples of nonviral approaches that are as effective in the process of gene transfer (39). Even though the vast majority of cancer clinical trials make use of modified cells and ex vivo techniques, in vivo studies have also made significant progress, particularly in the utilization of human herpes simplex virus (HSV) as an oncolytic virus. Imlygic, also known as Talimogene Laherparepvec, is a genetically modified herpes simplex virus that was granted approval by the Food and Drug Administration (FDA) in 2015 as the first oncolytic virus utilized for the treatment of metastatic melanoma (40). There are currently several gene therapy products that have been granted permission for the treatment of cancer. These medicines include Oncorhin, Rexin-G, and CAR T cells (41).

Gene Therapy and Breast Cancer

Breast cancer is the most common form of cancer and

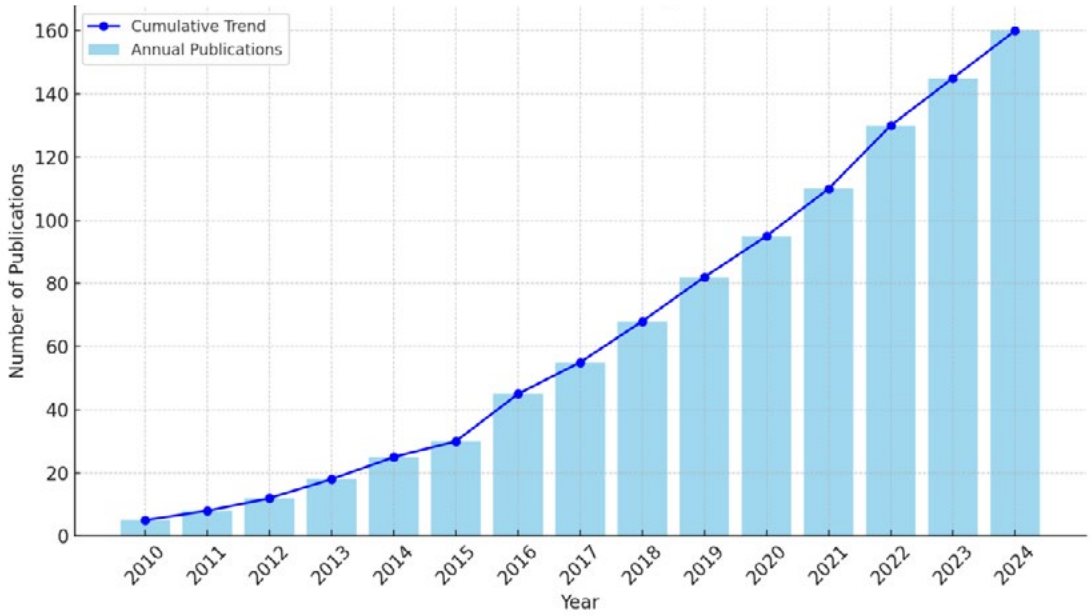

Fig 1. Number of Pepers Published on Al in Gene Therapy for Breast Cancer (2010-2024).

the second leading cause of death among women as a whole across the world. According to the most recent data from the national level, the incidence rate of breast cancer, when adjusted for age, is 21.33 per 100,000 people (42). Treatment approaches that are considered to be typical for breast cancer include surgical procedures, radiation therapy, chemotherapy, and hormone therapy. Both drug resistance and considerable adverse effects contribute to the limited success of these treatments, which is further aggravated by the low selectivity of pharmacological therapy (43). Consequently, because inherited changes in these genes are a contributing factor in the progression of this disease, gene therapy was considered as a potential treatment for it. Given that they are involved in the process of repairing damaged DNA, the BRCA1 and BRCA2 genes have been shown to have a significant association with breast cancer (44, 45). It is estimated that women who have a positive BRCA1 test have a probability of developing breast cancer by the age of 17 which ranges from 41 to 78 percent (46). By the year 2008, a prospective cohort study projected that those who carried the BRCA1 mutation had a cumulative breast cancer risk of 72%, while those who carried the BRCA2 mutation had a risk of 69% (20). There are around 2,000 variants that have been found in these two genes, and some variants in particular areas of both genes have been associated with an elevated risk (47, 48). In addition to these two genes, several other genes, including STK113, PTEN2, TP, and 14NF, have also been known to be associated with this type of illness (49). The processes involved in gene therapy for breast cancer are described in this document. Gene modification is a procedure that includes replacing a normal gene with a mutant gene. This technique, when combined with other cancer treatments such as chemotherapy or radiotherapy, has the potential to eliminate cancer (50, 51). Within the revolutionary methodology known as the gene transfer method, the equipment that is responsible for gene transfer directly penetrates the target cell without making use of a vector (52). This strategy makes use of nuclease enzymes, which, upon binding to the target DNA, cause double-strand breaks to occur (53). As was mentioned earlier, one of the methods for gene transfer is the utilization of genes that are responsible for suicide. Using this method, genes such as cytosine deaminase and herpes simplex virus thymidine kinase are introduced into cancer cells. These genes then cause the cancer cells to create enzymes that convert safe prodrugs into harmful metabolites, which ultimately results in the death of cancer cells (54). Inhibiting gene transcription and translation is the goal of the gene suppression approach, which ultimately results in the suppression of oncogenes associated with cancer. This technique makes use of antisense oligodeoxynucleotides, which are primarily concerned with transcription, in conjunction with specific 5-SiRNAs (42). To target transcription factors using decoy oligodeoxynucleotides, double-stranded oligodeoxynucleotides are utilized. These oligodeoxynucleotides encode transcription factors and completely suppress gene transcription (55). By either accelerating metastasis or blocking the roles of tumor suppressors, certain microRNAs, which are referred to as onco-microRNAs (for example, miR-21 and miR-92), act as tumor promoters. On the other hand, other microRNAs, such as miR-34, act as tumor suppressors (56, 57). Breast cancer can be caused by either of the two groups of microRNAs. In

light of this, targeting microRNAs is considered to be a gene therapy technique for treating this variety of cancer. Aptamers are single-stranded DNA or RNA oligonucleotides that are relatively short and possess a high level of sensitivity for genetic targeting. Aptamers can specifically target cancer cells and promote gene transfer by acting as vectors that are not the result of viral infection (58).

The method of vaccination involves injecting DNA directly into the body of the patient, which results in the production of antigens that are associated with tumors. Considering that breast cancer is a systemic condition that has the potential to spread to other parts of the body, this method is effective (59). According to research published by the International Agency for Research on Cancer in the year 2020, breast cancer has quickly become the most prevalent form of cancer worldwide (60). Around 2.62 million new cases of breast cancer were diagnosed around the world, and around 685,000 people lost their lives as a result of the disease (61). Despite the multitude of breakthroughs that have been made in treatment approaches for breast cancer, the prevalence of breast cancer increased by 24.3% in the year 2020 in comparison to the year 2012 (62). Even though certain studies have shown that Burley gene therapy in breast cancer treatment has the potential to produce positive results, the evidence that is now available regarding its usefulness is still inconclusive. As a result, additional clinical trials are required to improve both its safety and its performance. According to a recent article, the recurrence rates of breast cancer after five years were: seven percent, eleven percent, and thirty-one percent for patients in stages one, two, and three, respectively (63). There are variations in the life expectancy rates of breast cancer patients around the world, with survival rates being higher in more developed countries compared to less developed countries (64). These statistics unequivocally illustrate the imperative for revolutionary approaches, including the application of artificial intelligence in innovative treatment methods such as gene therapy (65).

Overview of AI Application in Cancer

This enables the difference between healthy and unhealthy individuals, as well as the prediction of treatment efficacy in patients. Artificial intelligence algorithms have the potential to evaluate enormous data sets and uncover subtle patterns (74). The capabilities of this field in terms of analysis are especially useful in the field of oncology. For instance, if an algorithm that is supported by artificial intelligence can predict how a patient will react to chemotherapy before the treatment is administered, this could make it possible for doctors to personalize treatment plans for patients (75). Dose modification may be one of the powers of artificial intelligence, which would allow medical professionals to provide systemic medicines with increasing doses that are based on AI predictive models (76, 77). A study that was conducted in 2012 suggests that artificial intelligence has the potential to revolutionize the production of nanofactors for gene therapy and mRNA vaccines, which could have a positive impact on the treatment of cancer (78).

More effective treatments for a variety of cancers could likely be developed through the utilization of artificial intelligence to optimize the design and delivery of these medicines. It has been suggested by research that the combination of artificial intelligence with multifunctional magnetic nanostructures could potentially improve the effectiveness of cancer treatment (79). The findings of this study demonstrate that nanostructures can be injected with medications and then supplied directly to cancer cells. This process makes it possible to develop a more individualized treatment plan that has fewer negative side effects (80). Moreover, they have the potential to be exploited in imaging modalities, which can assist medical professionals in the early diagnosis of cancer. The study, on the other hand, advocates for the application of artificial intelligence to enhance the utilization of these nanostructures (81). This is because the nanostructures have the potential to be toxic to healthy cells when they are present in high concentrations, and their effectiveness is influenced by parameters such as size and morphology (82). There are several applications for artificial intelligence, including the prediction of the efficacy of particular treatment modalities, the enhancement of dosage levels, and even the development of more effective nanostructures (83).

Application of Artificial Intelligence in Breast Cancer Gene Therapy

It is primarily due to genetic and molecular anomalies that result in varied tumor morphologies, which ultimately influence tumor responses to cytotoxic medications, that the heterogeneity of breast cancer provides a substantial barrier to the treatment process (84). As of right now, a great number of genetic and molecular factors that influence oncogenes have been identified (85). These factors include genes that control proliferation, the cell cycle, invasion, and metastasis. The field of breast cancer research is beginning to recognize the potential benefits of (AI). The application of artificial intelligence techniques is allowing researchers to make progress in the areas of breast cancer risk assessment, gene therapy, and personalized medicine (86). Emerging fields of medical imaging that make use of artificial intelligence algorithms for the noninvasive study of breast cancer tumors are making it possible to provide patients with treatment options that are more individualized and effective (87). It has

been demonstrated that several artificial intelligence models, such as artificial neural networks and decision trees, are capable of accurately predicting the risk and probability of developing breast cancer based on the presence of BRCA gene mutations (88). According to thirty, these models can be of use in determining the treatment method that is most suitable for a specific genetic profile (89).

Selecting the best genes with AI

Different molecular characteristics are possessed by each patient, which results in a wide range of responses to treatment (90). Because of the accumulation of unique mutations, which leads to heterogeneity, which makes diagnosis and therapy more difficult, the variation among patients is especially obvious in the various forms of cancer (91). With the use of several genetic and epigenetic markers, personalized medicine aims to tailor treatment plans to the specific needs of individual patients. To examine a patient's various markers, researchers in the field of tailored medicine use complex algorithms that make use of machine learning and artificial intelligence (43).

This research finds several genes that are potentially responsible for the development of breast cancer in an individual. Through the use of this knowledge, researchers can develop gene therapies that are substantially more accurate and effective than the approaches that have been traditionally used. One advantage of AI-driven tailored medicine is the mitigation of risks, such as off-target effects, which could result in severe consequences. This renders gene therapy potentially safer and more efficacious than it would have been otherwise (92). Through the examination of gene expression patterns derived from breast cancer tumor samples, a study conducted in 2020 indicated that out of the 34 genes and 43 transcription factors that were presented to artificial intelligence, 17 genes, including AMELX1 and FREM12, were identified as prognostic biomarkers for breast cancer (93). A 2020 study analyzing gene expression patterns from breast cancer tumor samples revealed that, among 34 genes and 43 transcription factors evaluated by artificial intelligence, 17 genes, including AMELX1 and FREM12, were recognized as prognostic biomarkers for breast cancer. Artificial intelligence often identifies a greater number of biomarkers, resulting in enhanced personalized medicine outcomes. Furthermore, it can identify intricate patterns and correlations between certain genetic modifications and clinical results (94).

Targeted therapy based on personalized medicine with the help of artificial intelligence

In a study that was conducted in 2013, three pathologists evaluated patients using immunohistochemistry to determine whether or not they had HER2 status. Following this, the researchers utilized thematic algorithms and trained an artificial intelligence model on a dataset consisting of breast cancer cases to determine the HER2 status of primary and metastatic tumors (95). A comparison was made between the results of the AI and the evaluations of the pathologists, which revealed that the AI model successfully and properly diagnosed the HER2 status of all types of breast tumors (96). This study's findings indicate that artificial intelligence enhances the precision and efficiency of HER2 status assessments, resulting in more effective therapy alternatives for these patients. A separate study found that artificial intelligence models can accurately forecast the risk of breast cancer in patients with BRCA mutations (97). This study indicates that artificial intelligence can improve the accuracy of breast cancer treatment by enabling therapeutic targeting based on an individual's genetic profile in customized medicine. Furthermore, the amalgamation of AI with person-centered oncology has demonstrated enhanced precision in therapeutic efficacy (98).

The algorithms that are used for machine learning can analyze vast amounts of patient data and identify groups of people who share similar genomic patterns. This classification of genomic information allows medical professionals to design treatments for specific patients depending on the genetic traits of those patients, which ultimately results in a treatment plan that is more personalized and effective (98). In light of this, person-centered oncology and artificial intelligence have the potential to revolutionize the treatment of breast cancer. The identification of biomarkers and the accuracy of treatment planning may lead to the development of more effective medicines and for patients to experience more positive clinical outcomes (99).

DISCUSSION

The purpose of this review is to provide an overview of gene therapy and its various approaches in the treatment of breast cancer patients. These approaches include gene modification, gene editing, the use of suicide genes, gene silencing, the targeting of transcription factors through the use of decoy oligodeoxynucleotides, the targeting of microRNA, the targeting of breast cancer cells through the use of aptamers, and vaccination with DNA or RNA (100).

Performance level in the world

In this study, a research pathway is shown, which was accomplished through the utilization of Google Scholar and a general search of pertinent phrases. The results of the search have been compiled into Table 1, which contains an illustration of the significance of this subject matter due to the considerable volume of publications from around the world (101). In oncology research, applications include identifying predictive

biomarkers by analyzing gene expression patterns in breast cancer samples, accurately classifying HER2 status across different breast tumor types, and developing advanced medical imaging techniques that utilize artificial intelligence algorithms to analyze and interpret breast cancer tumors (103). Nonetheless, there are other constraints related to the application of artificial intelligence. These constraints encompass ethical challenges (such as obtaining informed consent for data consumption), potential data loss, substantial maintenance costs, and the necessity for regular software updates (104). The implementation of artificial intelligence may enhance societal health; nevertheless, due to existing limitations, its application must be approached with prudence. Artificial intelligence is employed for the accurate and timely diagnosis of genetic disorders, in addition to gene therapy for breast cancer (105, 106).

Adaptations and challenges in current methods

The real-world clinical validation of AI models is a critical challenge. Despite the exceptional efficacy of AI algorithms in many studies, these algorithms need to undergo clinical validation with extensive datasets before their incorporation into clinical practice. Recent endeavors in clinical validation, especially those concentrating on treatment outcomes, sometimes face limitations due to retrospective designs that may bring unforeseen biases, highlighting the necessity for prospective research (107). These prospective studies are essential for thoroughly understanding the effects of AI implementation on clinical practice and ensuring that AI tools are useful and dependable in a clinical environment (108).

Secondly, the incorporation of AI models into practical clinical environments entails issues that extend beyond validation, including utility and usability. To ensure utility, AI models must undergo thorough validation using randomized controlled trials that evaluate several clinical objectives (109). These outcomes must encompass overall survival, illness control, toxin mitigation, enhanced quality of life, and reduced healthcare resource consumption. AI models must undergo evaluation in practical environments to assess time efficiency, user satisfaction, and the acceptance of AI recommendations (110). The incorporation of a feedback mechanism through post-market surveillance is essential for detecting potential shortcomings and avenues for improvement, thereby ensuring the continuous advancement of these models. Real-time monitoring tools for healthcare professionals and AI algorithm developers are crucial to ensure the safe delivery of services. Moreover, the smooth integration of AI technologies with existing workflows, such as PACS, is essential for their efficient utilization (111).

A third problem is the generalizability or robustness of the AI model, which pertains to its consistent performance across diverse datasets, including the training dataset. Various techniques to tackle this issue involve utilizing datasets encompassing a diverse range of preanalytic and analytic parameters to improve model resilience; however, obtaining large-scale datasets with manual annotations poses obstacles in the creation of AI algorithms (112). To resolve this issue, various methodologies, like unsupervised learning and Generative Adversarial Networks, are being employed. Another obstacle that emerges is the potential misrepresentation of health issues in minority communities, due to the development of AI models predominantly based on data from majority populations. This circumstance may intensify health disparities (113, 114).

Fourth, many AI algorithms are frequently regarded as black boxes because of the ambiguity around the identifiable features within them. The development of explainable AI systems could foster trust among clinicians, enhance transparency in decision-making, and mitigate various biases (115). Conversely, Ghassemi et al. proposed that fervent internal and external validation of AI systems may provide a direct method for attaining objectives related to explainability (116).

Ultimately, reimbursement difficulties must be addressed, especially if AI systems begin to supplant specific functions previously executed by physicians. Discussions regarding reimbursement and the influence of AI on healthcare systems should occur at the national or screening program levels to guarantee equitable and successful implementation (117). To foster discourse and engagement among physicians, the wider medical team, pertinent governmental authorities, and hospitals regarding the integration of AI in healthcare, it is crucial to analyze the primary issues (118).

CONCLUSION

Consequently, in light of the data and the increasing prevalence of this cancer, along with the anticipation that breast cancer will emerge as the most common cancer type in 2024, the imperative for the use of fresh technologies and methodologies in treatment strategies was underscored. Gene therapy is profoundly impacted by artificial intelligence, a relatively emerging field of study. The field of artificial intelligence focuses on developing intelligent machines that can replicate human behavior through diverse learning and decision-making processes with limited external input. This research is applicable in differentiating between healthy and diseased persons, forecasting drug efficacy in patients, formulating personalized medicines, executing gene therapy, and evaluating breast cancer risk, all of which may improve community health outcomes. The advantages of engaging in a profession in intelligence

science Artificial intelligence can distinguish between healthy and unwell persons, forecast drug efficacy in patients, facilitate individualized therapies, aid in gene therapy, and evaluate breast cancer risk, potentially improving the overall health of the population.

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Prevalence and Potential Zoonotic Risk of Campylobacter Species in Dairy Cattle from Golestan Province, Iran

Ali Bahoosh[1], Ezzat Allah Ghaemi[1], Abdollah Ardebili[1], Sayeh Ghaemi[2], Ailar Jamalli[1*]

[1]Department of Microbiology, Faculty of Medical Sciences, Golestan University of Medical Sciences, Gorgan, Iran.
[2]Faculty of Veterinary Sciences, Islamic Azad university of Babol, Iran.

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Corresponding author:
Ailar Jamalli, Department of Microbiology, Faculty of Medical Sciences, Golestan University of Medical Sciences, Gorgan, Iran.
Email: jamali@goums.ac.ir.

ABSTRACT

Background: Campylobacter is the primary bacterial agent responsible for gastrointestinal (GI) infections in humans. Domestic animals, including cattle, are reservoirs of this bacterium and can be one of the main sources of infection transmission to humans. This study aimed to investigate the prevalence of Campylobacter species in cattle in the Gorgan province.
Materials and Methods: A total of 200 fecal samples were collected from healthy dairy cattle and genus and species were identified using multiplex PCR.
Results: The frequency of the genus Campylobacter in 200 samples in our study was 17.5% (35 cases), C. jejuni and C. coli species were not identified in these 35 cases.
Conclusion: Isolating Campylobacter from animal fecal samples is a challenging process, but this study showed that Campylobacter contamination was relatively high in cattle in the Gorgan province, and its transmission to humans through meat consumption must be monitored.

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INTRODUCTION

In recent years, the number of foodborne illnesses caused by *Campylobacter spp*. in humans has increased dramatically. According to the World Health Organization (WHO), *Campylobacter* is one of the four major causes of diarrheal diseases worldwide (1). *Campylobacteriosis* is a foodborne disease that significantly affects human health and life and can have economic consequences for individuals, families, society, and governments (2). Because *Campylobacter ssp*. Constitute the natural microflora of the digestive tract of livestock and wild animals, these bacteria are widely distributed in the environment and are isolated from various sources, including water, soil, and food (2). Food animals such as poultry, cattle, sheep, pigs, and ostriches. Pets, including dogs and cats, are environmental sources of Campylobacteriosis in humans (3). *Campylobacter* infection has been reported through the consumption of undercooked poultry, raw meat, and even milk and its products (4). These bacteria, which live in the digestive tracts of many warm-blooded animals, are excreted in approximately 20% of cattle feces (5). Campylobacter ssp are small (0.2–0.9 μm wide and 0.2–5.0 μm long) gram-negative

rod-shaped, spiral, curved, bird-wing, and gull-wing shapes and do not form spores (6). The movement of this bacterium is corkscrew-like. *Campylobacter* is a microaerophilic bacterium that requires specific conditions (10% CO2, 5% O2, and a temperature of 37-42°C) for growth (7). Pathogenic Campylobacters associated with human infections include *C. jejuni, C. concisus, C. rectus, C. hyointestinalis, C. insulaenigrae, C. sputorum, C. helveticus, C. lari, C. fetus, C. mucosalis, C. coli, C. upsaliensis, and C.* ureolyticus (8). *C. jejuni* and *C. coli* are the most common zoonotic diseases in humans and the most common cause of bacterial gastroenteritis worldwide (9). In humans, clinical symptoms of campylobacteriosis are mild to moderate with gastrointestinal symptoms, and in some cases, more severe diseases may occur such as Guillain–Barré syndrome (10). Unlike humans, *Campylobacter* infections in animals are often asymptomatic (11). Emerging *Campylobacter spp*. are currently overlooked, but the integration of molecular techniques and appropriate culture media into current diagnostic tests will help improve awareness of unusual species as relevant human and animal pathogens (12). Indirect exposure to cattle feces through environmental contamination is considered a high risk to humans (13). The longer lifespan of dairy cows compared to beef cattle could lead to permanent or prolonged shedding of *Campylobacter* by dairy cows, which act as long-term reservoirs (14). Therefore, the role of cows as reservoirs of *Campylobacter* species is important for understanding the epidemiology of this pathogen. This study aimed to investigate the prevalence of *Campylobacter* infection in dairy cows using molecular methods.

MATERIALS AND METHODS

Sample collection

The medical ethics code for this study was IR.IAU.BABOL.REC.1400.131. In this descriptive cross-sectional study, 200 feces samples were collected from November 2021 to the end of March 2022. All samples were taken from female animals that were healthy cows. The ages of the cows studied ranged from 2 to 8 years. The consistency of the collected feces also ranged from one to five, with most of them having a consistency of three (normal feces).

Molecular identification of Campylobacter spp

DNA extraction from the stool

The stool samples were transported to the microbiology laboratory using an ice pack. Due to the difficulty of culturing and biochemical methods for identifying *Campylobacter spp*., Stool DNA was extracted using a DNA extraction kit manufactured by Favorgen.

Multiplex PCR was used for the molecular identification of Campylobacter species. Primers used in this study are listed in Table 1. The total PCR reaction volume was 25 µL, comprising 12.5 µL of PCR master mix (1×), consisting of Taq DNA polymerase (0.06 U/µL), MgCl2 (1.5 mM), dNTPs (0.2 mM),1 µL each of the F and R primers, 1 µL of template DNA, and 8.5 µL of sterile deionized water. All the primers used in this study were synthesized by the German Metabion Company. The cycling conditions were as follows: initial denaturation at 95°C for 10 min; 25 cycles of 94°C for 45 s, 58°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 7 min. PCR products were electrophoresed on a 1.5% agarose gel mixed with 0.4 µl SYBR Green dye and photographed by UV irradiation.

Data Analysis

Statistical analysis was conducted using the SPSS software (version 26.0). The Chi-square test and ANOVA method were employed to calculate P values. Results were deemed statistically significant when P <0.05, across all instances.

Table 1. Primers used to detect *Campylobacter* genus and species

Species	Gene		Sequence (5′ to 3′)	Size (bp)	Ref
Genus *Campylobacter*	*16s rRNA*	C412F	5′-GGATGACACTTTTCGGAGC-3′	816	(15)
		C1228R	5′-CATTGTAGCACGTGTGTC-3′		
C. jejuni	*cj0414*	C-1	5′-CAAATAAAGTTAGAGGTAGAATGT-3′	161	(16)
		C-3	5′-CCATAAGCACTAGCTAGCTGAT-3′		
C. coli	*ask*	CC18F	5′-GGTATGATTTCTACAAAGCGAG-3′	502	(17)
		CC519R	5′-ATAAAAGACTATCGTCGCGTG-3′		

RESULTS

In this study, 200 fecal samples were collected from healthy dairy cows, 25 from traditional livestock farms, and 175 from industrial livestock farms. The age of the sampled dairy cows varied between 2 and 8 years, and the consistency of the cows' feces also ranged from one to five, with 133 samples (66.5%) having consistency of three (normal feces), 4 (2%) had loose consistency, 61 (30.5%) had medium consistency, and 2 (1%) had hard and lumpy consistency. In this study, out of a total of 200 cow feces samples, 17.5% (35 samples) contained bacteria belonging to the genus *Campylobacter*. In these 35 samples, no samples with bands related to *C. jejuni* and *C. coli species* were observed (Figure 1).

The frequency of *Campylobacter* in the feces of cows aged 2, 3, 4, 5, 6, 7, and 8 years was 20.4% (10 cases), 18.8% (13 cases), 8.3% (3 cases), 11.5% (3 cases), 18.1% (2 cases), 42.8% (3 cases), and 50% (1 case), respectively. The highest percentage of positive cases by age was in the seven and eight age groups, which were 42.8% and 50%, respectively. However, the percentage increased in the seven and eight age groups, which, given that the number of samples in this case was only seven and two samples, may be due to the limited number of samples. In the chi-square analysis, there was no significant relationship between the level of infection in the livestock and age. Additionally, based on ANOVA analysis, the mean age of positive cases was 3.6±1.7, and the mean age of negative cases was 3.5±1.3 (P=0.7), and there was no significant relationship between age and the frequency of *Campylobacter* infection (Figure 2).

In campylobacteriosis, stool appears watery (profuse); due to the limited number of watery and loose stool samples, all samples were divided into two categories: normal (normal, medium, and hard stool) and abnormal (watery and loose). The results showed that all *Campylobacter*-positive cases were in the normal stool group, and none of the abnormal samples were positive. Using the chi-square test, no statistically significant relationship was found between positive cases and stool scores (P=0.46) (Figure 3).

Out of 200 samples, only 4 were abnormal, but *Campylobacter* was negative (p=0.4). There was no significant relationship between the frequency of *Campylobacter* and the clinical signs of the cows. 25 samples were collected from local farms and 175 samples from industrial farms, of which 8 (32%) samples from local farms and 27 (15%) from industrial farms were positive for *Campylobacter* (p=0.04). There was a significant relationship between the frequency of *Campylobacter spp.* and the farm type.

DISCUSSION

Worldwide, the leading cause of foodborne illness is caused by pathogenic *Campylobacter spp*. Accounting for more than 400 to 500 million infections per year (18). *Campylobacter* species are commonly found in the intestinal tract of many domestic animals, such as poultry, cattle, sheep, goats, pigs, wild animals, and birds (19). Feces are the main source of contamination (20) and animal food products can be contaminated with this pathogen during slaughter and cutting of carcasses (21). *Campylobacter* is the leading cause of gastroenteritis in humans worldwide (22). Recently, *Campylobacter* species isolated from human and

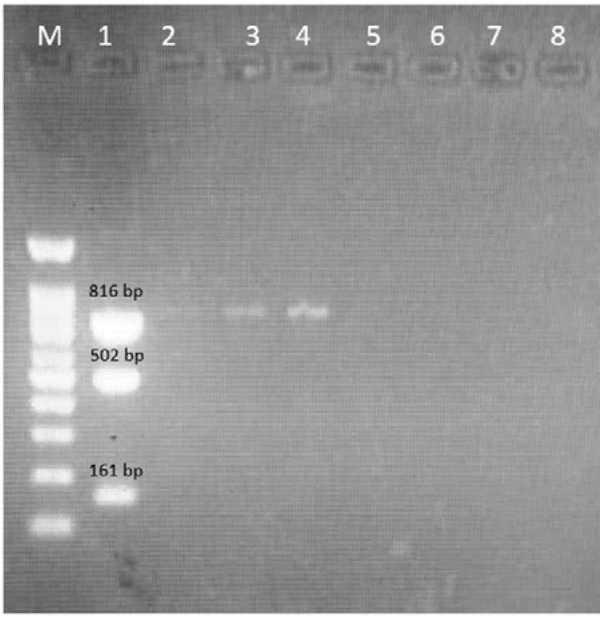

Fig 1. PCR product of feces samples from healthy dairy cows using primers specific for the genus and species of *Campylobacter* on agarose gel; M, 100bp molecular weight marker, Lanes:1, positive control containing the genera and species of *C. jejuni* and *C. coli*, Lanes 2-4: positive samples; Lanes 5- 7: negative samples; Lanes 8, negative control.

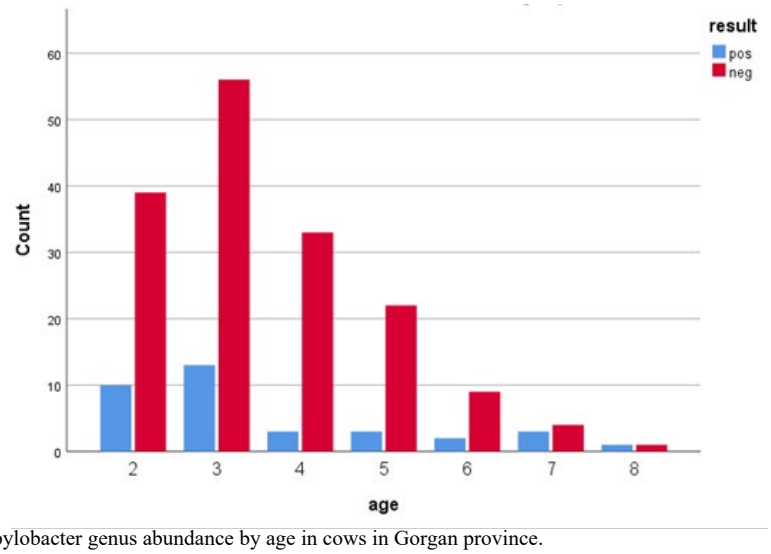

Fig 2. Distribution of Campylobacter genus abundance by age in cows in Gorgan province.

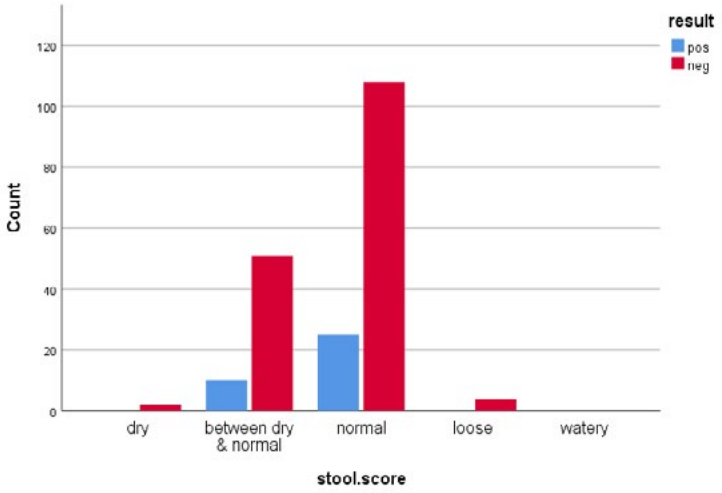

Fig 3. Frequency of *Campylobacter* genus according to clinical symptoms in healthy cows in Gorgan province

animal samples have shown increased resistance to various antibiotics (23). However, the epidemiology of *Campylobacter* remains unclear in many countries (24). In the present study, which was conducted in connection with the investigation of the frequency of *Campylobacter* species in cows in Gorgan province using the Multiplex PCR method, 17.5% Campylobacter species were isolated from a total of 200 samples examined, and none of the *C. jejuni* or *C. coli* species were observed in the samples. In 2021, Hagos et al. reported the prevalence of *C. jejuni* and *C. coli* in 384 meat samples, including 210 beef, 108 goats, and 66 chicken meat samples. Molecular methods reported 64 cases (16.67%), with a prevalence of 11.9% in beef (25). The level of contamination in beef in this study was lower than that in our study. Kagambega et al. in 2021 reported the abundance of *Campylobacter* species in 52 chicken feces samples, 18 (34.61%) of which 17 (94.45%) were *C. coli* and 1 (5.55%) were C. jejuni (24). No *C. coli* or *C. jejuni* species were isolated in this study. In 2021, Gahmanyie et al. conducted a study aimed at molecular detection of *Campylobacter* spp. in 70 human and 30 bovine stool samples. Of the 70 human stool samples, 65.7% were *Campylobacter,* and of the 30 bovine stool samples, only 20.6% were *Campylobacter* positive (26). The difference between the results of the present study and previous studies may be attributed to various factors, including adherence to hygiene principles from the time of raising livestock on the farm to the various stages of slaughter in the slaughterhouse, the prevalence in livestock herds, and climatic conditions. Igwaran et al. conducted a study in 2020 on 1238 samples with the aim of molecular identification of *C. jejuni* and *C. coli* isolated from milk and beef. 71 samples were positive by PCR, of which 35 were positive for *C. coli* and 36 for *C. jejuni* (27). Nevertheless, there are few studies documenting the occurrence of *Campylobacter* in both human and animal populations, likely because, since most healthcare facilities and veterinary clinics in developing nations do not routinely test for this pathogen (26). The reason for the difference in the prevalence of

Campylobacter in cattle can be influenced by several factors, such as season, stress, livestock management factors, diet, and immune system. The reason for this difference in the identification of *Campylobacter spp.* in our study is unknown, but one of the reasons could be the sampling season of cattle, which was conducted in winter.

CONCLUSION

Campylobacter species are one of the main causes of gastroenteritis in humans, transmitted to humans by their vectors, including livestock, such as cattle, and by consuming its meat. In this study, we reported the level of contamination by these bacteria in healthy cows in Gorgan province. However, no common species, such as *C. jejuni* and *C. coli,* were observed. Given the emergence of antibiotic resistance in *Campylobacter,* which can be a warning sign for therapeutic purposes, it is necessary to investigate the epidemiology of the spread of this bacterium and closely monitor livestock farms as a source of transmission.

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Declarations

Authors' contributions

A. B. performed the experiments, analyzed the experimental data, and wrote the manuscript. E. GH and A. Contributed to the concept of the article. A. J guided the experiments and critically revised the manuscript.

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Consent for publication

Not applicable

Availability of data and materials

All of the data generated and analyzed during this study are included in our manuscript.

Conflicts of Interest

There are no conflicts of interest associated with this manuscript.

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