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

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Table of Content

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Cancer Immunotherapy Using Microfluidic Systems

Maryam Diansaei[1,*], **Parisa Sanati**[2]

[1]Department of veterinary medicine, Islamic Azad University of Tabriz, Tabriz,Iran.
[2]Burn and Wound Healing Research Center, Shiraz University of Medical Sciences, 71345-1978, Shiraz, Iran.

**Corresponding author: Maryam Diansaei, Department of veterinary medicine, Islamic Azad University of Tabriz, Tabriz,Iran.Email: maryam.diansaei@yahoo.com.*

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Abstract:

Physicians are enthusiastic about using a novel approach known as cancer immunotherapy to address various forms of cancer. However, there are occasions when novel therapies that demonstrate efficacy in laboratory settings may not provide the same level of effectiveness when applied to actual patients. To address this issue, scientists are using miniature replicas known as microfluidic models. These models provide the examination of the interaction between cancer and immune cells in a manner that closely resembles the physiological conditions inside the human body. This review examines the role of microfluidic models in advancing the development of more effective cancer therapies. Let's begin by discussing the current state of affairs in cancer immunotherapy. Next, we explore the use of microfluidic models by scientists to gain insights into the mechanisms via which the immune system combats cancer and to evaluate the efficacy of novel therapeutic interventions. Additionally, we discuss the first measures used to demonstrate the efficacy of these models in predicting the effectiveness of therapies in human subjects. Lastly, we will discuss the advantages of using microfluidic models and the necessary steps to enhance their efficacy in the development of novel cancer therapies.

INTRODUCTION

Globally, cancer continues to be the primary cause of mortality, even with the widespread acceptance of numerous innovative treatments—certain of which have demonstrated enduring curative advantages. Recently, there has been a significant change in the way cancer is treated with the advent of cancer immunotherapy. This revolutionary plan utilizes the immune response to battle and potentially eliminate malignant cells, thereby transforming the chemotherapy landscape. Corresponding

with this, immunotherapy is presently a quickly expanding discipline in the medicinal management of cancer, with antibody-based and cell-based methods constituting the most prominent examples. Monoclonal antibodies (mAbs)., that attack checkpoint molecules or cancer-associated markers (e.g., CD20, EGFR, HER-2, CD38)., make up more than fifty per cent of the more than one hundred antibodies presently accessible for daily clinical use. This class of cancer (immuno).therapies is crucial and increasing rapidly (1).

Developments in antibody engineering have facilitated the production of new compounds based on antibodies that have specific effector activities or modified pharmacokinetics (2). Immune checkpoint-blocking antibodies, which commonly inhibit inhibitory receptors on T cells (e.g., PD-1 and CTLA-4)., have created considerable interest in the recruitment of patients' T cells to combat tumor cells (3). In addition to classical T cell-recruiting checkpoint blockers, immune-related checkpoints on myeloid effector cells that inhibit antibody-dependent cell-mediated cytotoxicity (ADCC). by neutrophils or antibody-dependent cellular phagocytosis (ADCP). by macrophages were also identified. Although the CD47/SIRPα axis represents the most notable and clinically advanced instance, there are currently antibodies in development targeting numerous other molecules. Among the various classes of molecules comprising antibody-based therapeutics, bispecific antibodies (BsAb). are presently experiencing substantial growth (4). Numerous BsAb bound to CD3 and antigens expressed by tumor cells, thus recruiting and activating T cells specifically. Conversely, BsAbs that bind to Fc receptors (e.g., those targeting FcγRI). were also rapidly tested in clinical settings and could potentially reappear as a novel method to recruit myeloid effector cells, including dendritic cells (DCs)., neutrophils, and macrophages (5).

Alongside bispecific antibodies and checkpoint blockers, chimeric antigen receptor (CAR).-transduced T cells are also considered a key component of authorized T cell-recruiting immunotherapies. Typical CAR constructions consist of an external single-chain antibody that is linked to intracellular activation domains (such as CD3ζ). and costimulatory domains (such as CD28 or 4-1BB) (6). CARs exhibit variations in the duration and strength of T cell activation, as well as their longevity in patients, depending on the signaling domains involved. The currently authorized target antigens for CARs include CD19 (four products). and BCMA (two products)., with the anticipation of more to come, such as CD22. The usual therapeutic indications mostly target blood cancers, whereas advancements in treating solid tumors are hindered by problems related to toxicity (7).

In addition to CAR T cells, additional modified cell products are being created, with encouraging outcomes in early clinical trials. As an alternative to CAR-T, engineered T cell receptor (TCR).-expressing T cells (TCR-T). have been proposed for solid tumor therapy. Baulu et al. examined recent clinical data for TCR-T treatments (8). Natural killer (NK). cells constitute an alternate cell therapy source that has the potential to overcome some of the drawbacks of CAR T, such as cytokine release syndrome and neurotoxicity. Currently, allogenic NK cell products are being studied in clinical studies, with encouraging findings in patients with acute myeloid leukemia and other hematological malignancies (reviewed by Berrien-Elliott et al) (9).

Aside from antibody-based and cell-based techniques, vaccinations against human papillomavirus and hepatitis B were effective in preventing cervical and liver cancer, respectively (10). Following the success of mRNA-based vaccinations in preventing Covid-19 infections, individualized therapeutic tumor vaccines show potential for treating pancreatic cancer (11). or melanoma (12). Furthermore, the use of modified oncolytic viruses capable of targeting and reproducing cancer cells while causing no harm to healthy cells has increased in recent years (13).

Microfluidics in biomedicine

Despite the considerable potential of cancer immunotherapies, the development of new ones continues to be a complex, time-consuming, and expensive endeavor, accompanied by a scarcity of approvals. Market analysts estimate that a solitary oncology agent could cost as much as $4.5 billion (14, 15). This necessitates the development of “fast failure” solutions which allow the early detection of ineffective medications or their repurposing (16). Due to the inadequate transfer value of preclinical models, which frequently neglect to faithfully replicate the human tumor microenvironment (TME)., the advancement of cancer immunotherapies is extremely expensive. In contrast to conventional two-dimensional cell culture models, which fail to incorporate critical attributes such as three-dimensional morphology, extracellular matrix, gradients, physiological oxygen levels, and vasculature, animal models have several drawbacks. These include inadequate productivity and turnover and high costs, a failure to dissect and regulate processes within the tumor microenvironment (TME)., and rodent models, in particular, possess immune systems that are significantly distinct from the human immune system (17).

Hence, microfluidic models provide a convincing substitute for conventional in vitro and animal models. These models provide a distinct benefit by faithfully reproducing intricate physiological microenvironments, while also allowing for dynamic and real-time examination of cellular responses. Furthermore, the natural capacity of microfluidic platforms to easily adjust in size, their decreased need for large sample sizes, and their capability for conducting experiments at a rapid rate make them very significant instruments for improving research in the field of biomedicine.

Microfluidics originated in the 1980s and is now a quickly advancing discipline that provides valuable tools for connecting traditional in vitro models with in vivo models. Microfluidics involves the manipulation of fluids on a very small scale, often in the range of tens to hundreds of micrometres. This is achieved using tiny systems that include linked channels, chambers, and reservoirs. This enables the manipulation of flow and molecule concentrations with greater precision by operating at low Reynolds numbers, resulting in laminar flow. The primary focus of biomedical research is to faithfully replicate the physiology of tissues in organs-on-chips, which is a crucial technology for developing tissue models that correctly simulate the 3D tissue environment (18).

Microfluidic devices can be manufactured using a variety of techniques and materials (e.g., etching, molding, soft lithography, 3D printing)., including silicon, plastic, paper, glass, and polydimethylsiloxane (PDMS).. PMADS continues to be the most extensively utilized material in the fabrication of microfluidic devices. PDMS is a readily accessible and inexpensive material. The material’s optical transparency facilitates on-chip high-resolution imaging, while its flexibility permits the fabrication of deformable elements and valves. Additionally, its biocompatibility and gas permeability establish it as an exceptional material for biomedical applications (19, 20).

However, a significant disadvantage of this material is its hydrophobic nature and its tendency to absorb

tiny biomolecules. As a result, researchers have been exploring and using other polymers including poly(methyl methacrylate). (PMMA).. Fang et al (19). recently conducted a comprehensive analysis of the manufacturing technologies that are often employed in tumor-on-a-chip systems.

Microfluidic devices enable a variety of functions, such as evaluating multiple variables, controlling gradients (including oxygen levels)., compressing and stretching the tissue, applying sheer stress and interstitial flow, and integrating sensors to measure physical and chemical properties (such as electrical signals, pH, and oxygen levels). These capabilities have been reviewed by Ko et al (20). and Palacio-Castañeda et al (21).

The combination of engineering and cell culture methods has led to the creation of microfluidic tumor models, often known as tumours-on-chips. These models provide a compartmentalized and more physiologically appropriate depiction of the tumor microenvironment (TME), which can be controlled. They are increasingly being employed for the development of immunotherapy. This research presents a summary of how the advancement of various sorts of immunotherapy (such as antibody- and cell-based treatments, cytokines, oncolytic viruses, and cancer vaccines). is enhanced by the utilization of microfluidic platforms. This text examines the potential, constraints, and future possibilities of using microfluidic models to produce cancer immunotherapies. It emphasizes the circumstances in which these models might aid in the translation of cancer immunotherapies from laboratory research to clinical application.

Development of cancer immunotherapy using microfluidic tumor models

In recent years, researchers have created and used many microfluidic tumor models to further the development of cancer immunotherapy. The majority of models consist of parallel channels, which typically include a tumor section containing a hydrogel that contains cancer cells or spheroids, as well as immune cells that are either embedded in a hydrogel or perfused from a side channel. The selection of the microfluidic model design is usually determined by the individual research topic being investigated. This is because the throughput, dynamic features (such as flow), and molecular readout options may vary significantly across different models. In this discussion, we will explore the latest uses of microfluidic models in the advancement of major forms of cancer immunotherapies.

T-cell immunotherapy

Microfluidic models are becoming essential for exploring T cell-based anti-cancer therapy. These models enable systematic studies of immune cell interactions and procedures for therapy.

T Cell-Based Monoclonal Antibody (mAb). Therapies

Breast and Colon Cancer: Jiang et al. created a high-throughput observation chamber (iHOC). to examine the impact of an anti-PD-1 antibody on T cells associated with breast cancer. This study showed that the antibody was able to counteract the suppression of T cells caused by PD-L1, increase the production of IL-2, and boost the ability of T cells to enter and survive inside the tumour microenvironment. Sehgal et al. used a commercially available microfluidic system to demonstrate that the combination of PD-1 blockage and Birc 2/3 antagonism enhanced the eradication of cancer cells in colon cancer spheroids (26, 27). Glioblastoma: Cui et al. developed a chip made of PDMS that combines patient-derived GBM cells with other immunological components. It was shown that combining PD-1 and CSF-1R inhibitors might effectively counteract immunosuppression in aggressive GBM, as evidenced by cytokine profiles that closely resembled those of actual patients (23).

Cell Therapies

Leukemia: Chen et al. constructed a microfluidic model that replicates a leukaemia niche to investigate CAR-T cell therapy. Their model precisely depicted clinical reactions, including remission, resistance, and recurrence. Additionally, it emphasised the aspects that contribute to the lack of success in therapy and proposed its use as a tool for pre-clinical trials (24).

Liver Cancer: Preece et al. used a three-lane chip model to evaluate the efficacy of TCR-engineered T lymphocytes against hepatoma cells. Their research demonstrated an increase in the production of cytokines and the ability of these modified T cells to destroy tumour cells, indicating the promising potential of these altered T cells (28).

Security and immune response to foreign cells Kerns et al. confirmed the validity of lung- and intestine-on-a-chip models for evaluating the safety of T cell bispecific antibodies (TCBs). Their models accurately forecasted the optimal time frames for effectively eliminating cancer cells while minimising injury to healthy organs, in line with the observed reactions in animal models. Although alloreactivity is a problem, the period and components used in these in vitro investigations often reduce this danger. To further reduce alloreactivity, it is important to ensure compatibility between HLA/MHC or use cells produced by the patient (29, 30).

Natural Killer (NK). Cell Therapies

Studying the actions of NK cells in microfluidic tumor models is essential for gaining valuable knowledge about their ability to fight cancer and their potential use in therapy.

Interaction between natural killer (NK). cells and cancer

Nguyen et al. and Marzagalli et al. investigated the function and characteristics of NK cells by using microfluidic models of colorectal cancer and neuroblastoma, respectively. Nguyen's model used functioning cardiac tissue to evaluate tolerability, demonstrating targeted tumor destruction by NK cells without inducing structural abnormalities, but also decreasing the beating rate of the heart tissue. Marzagalli's model specifically examined the process of NK cell migration and extravasation, demonstrating the selective recruitment of CD16-negative NK cells to neuroblastoma spheroids and subsequent induction of tumour cell death. These models play a crucial role in assessing the behaviour, movement, and effectiveness of NK cells in controlled settings (31, 32). Therapies use monoclonal antibodies to activate natural killer cells Gopal et al. employed a high-throughput microfluidic device to investigate the synergistic impact of trastuzumab (an anti-HER2 drug). and atezolizumab (an anti-PD-L1 drug). in combination with doxorubicin and/or paclitaxel on the ability of natural killer (NK). cells to destroy pancreatic and breast cancer cells. Their technique caused a state of low oxygen levels inside tumour spheroids and showed a decrease in the amount of chemotherapeutics needed to achieve 50% effectiveness when paired with NK cells and antibodies (33).

Ayuso et al. investigated the impact of atezolizumab and the IDO-1 inhibitor epacadostat on a breast cancer model taken from a patient. The model was created using a specially designed PDMS device. This chip used an endothelial cell-patterned channel to create a functioning vascular tube, while a hydrogel was used to construct nutrition and metabolite gradients. A study revealed that environmental stress hindered the functioning of NK cells, although this effect may be partly reversed by inhibiting PD-L1 and IDO-1 (34).

Additional monoclonal antibody therapies

Recent research has investigated the efficacy of monoclonal antibody (mAb). therapy in using additional types of effector cells, including patient-derived tissue-resident immune cells and monocyte-derived macrophages, in addition to T and NK cells.

Immune cells gathered from the patient

Ao et al. evaluated the efficacy of anti-PD1 antibodies in mouse and human mammary cancer models utilizing an ex vivo on-chip model that included patient-derived material. Their PDMS apparatus, which consisted of 960 flow units and 16 channels containing 60 wells each, enabled the examination of tumor aggregates in a high-throughput manner across 16 different treatment settings. The researchers assessed the vitality of cells, examined the inflammatory profiles, and analyzed the makeup of the tumor. The in vitro reactions of separated syngeneic murine mammary carcinomas precisely anticipated the in vivo reactions, but original tumor cells from patient-derived samples exhibited diverse reactions to PD-1 inhibition. Although there is no evidence of clinical response, this research confirmed the ability of on-chip models to predict preclinical results (33, 35).

Macrophages derived from monocytes

Researchers conducted a study on monocyte-derived macrophages in a breast cancer model called MDA-MB-468. They used a microfluidic technology that is suitable for high-resolution imaging and cytokine/RNA analysis to combine an anti-EGFR IgA with a CD47 checkpoint blocker. The combined treatment effectively stimulated M2 macrophages, leading to an enhanced ability to engulf cancer cells and an elevation in the production of pro-inflammatory cytokines TNFα and IL-6. Additionally, there was an upregulation of both M1 and M2 markers. This research emphasises the significance of macrophages in cancer immunotherapy (36).

Alternative Immunotherapeutic Methods

Microfluidic systems are used to investigate novel immunotherapies, such as cytokine targeting, oncolytic viruses, vaccinations, and RNA-based medicines.

Targeting Cytokines and Oncolytic Viruses

Targeting Cytokines: Es et al. conducted a study to examine the effects of pirfenidone (PFD)., a drug that inhibits fibrosis, on cancer-associated fibroblasts (CAFs). in a breast cancer model. They used a microfluidic chip with three lanes for their experiments. The administration of PFD resulted in a decrease in the production of immunosuppressive cytokines, leading to a reduction in both CAF and cancer cell migration and invasiveness (37). Mencattini et al. used the same framework to investigate the oncolytic properties of the vaccinia virus (OVV). in a lung cancer model. Their investigation using video-microscopy showed that immune cell recruitment was improved and immune-cancer cell contacts were extended, resulting in greater cancer cell death in the presence of OVV (38). Vaccines and RNA-based methods Kim et al. used bioprinting to construct a three-dimensional bladder model to examine the impact of the Bacillus Calmette-Guérin (BCG). vaccination. The administration of BCG therapy resulted in an augmentation in the production of inflammatory

cytokines and the migration of macrophages, suggesting a possible immunostimulatory impactn (39). Hong et al. used a PDMS-based chip to investigate the efficacy of miRNA-based treatment in a glioblastoma (GBM). model. The researchers discovered that extracellular vesicles (EVs). containing miRNA-124 effectively hindered the development and invasion of GBM (glioblastoma). cells. Additionally, these EVs repressed the polarization of M2 microglial cells, reduced the production of proteins that promote tumor growth, and facilitated the recruitment of NK (natural killer). cells. These findings align with the gene expression patterns seen in GBM cell lines obtained from patients (40).

Conclusions and Future Considerations

Microfluidic tumor models are transforming cancer immunotherapy by including functioning immune components to investigate treatments, namely monoclonal antibodies such as PD-1 inhibition (41). These models allow for accurate manipulation of microenvironmental circumstances and continuous monitoring of interactions between immune cells and tumor cells, providing useful insights for both established and innovative immunotherapeutic strategies. They show potential in enhancing the process of choosing patients and creating treatments that include several types of immune cells, such as NK cells and macrophages. Although there are benefits, there are still obstacles to overcome, like the complex process of manufacturing, the need for uniformity, and the verification of prediction accuracy. Future developments will prioritize enhancing model complexity by including more cell types and developing multi-organs-on-chip systems (42). Furthermore, efforts will be made to improve the regulation of oxygen and pH levels and to establish standardized techniques to facilitate wider implementation (21). By incorporating regulatory frameworks and the pharmaceutical industry's discovery pipeline, together with the use of in silico modelling, cancer immunotherapy research and development will be greatly advanced, leading to improved prediction capacities and considerable progress.

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Prevalence of Drug Resistance and Some Pathogenic Factors in Uro Pathogenic Escherichia Coli (UPEC) Strains Isolated from Patients with Urinary Tract Infection

Zainab thaer Alshubidi[1], Ali Neamati[2*], Masoud Homayouni Tabrizr[3]

[1]Master of Cellular and Molecular Biology, Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran.
[2]Associate Professor of Physiology, Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran.
[3]Associate Professor of Biochemistry, Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran.

**Corresponding author: Ali Neamati, Associate Professor of Physiology, Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran. Email: neamati.ali@gmail.com.*

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Abstract:
Uropathogenic Escherichia coli is one of the most important causes of urinary tract infections. These strains possess various virulence factors, including adhesins, toxins, and iron acquisition systems. Virulence genes are situated on mobile genetic elements or in specific regions of the chromosome known as pathogenicity islands. In this study, 375 clinical samples from male and female patients suspected of having urinary tract infections were collected in the hospitals of Dhi Qar, Iraq, during the period from June 1, 2019, to November 1, 2019. Following the collection of 100 samples, bacterial isolation, DNA extraction, and antibiotic sensitivity tests were conducted using the disc diffusion method with the selected antibiotics. The presence of papC, aer, fimH, hly, cnf-1, and afa class genes was investigated using multiplex PCR. The results indicated that the highest frequency among the genes was associated with the fim gene (98%). The aer, papC, cnf-1, hly, and afa genes were also detected, with frequencies of 52%, 30%, 18%, 13%, and 11%, respectively. Additionally, the highest resistance and sensitivity among UPEC isolates were observed for amoxicillin (82.37%) and amikacin (92.35%) antibiotics, respectively.

INTRODUCTION

The emergence of resistance in pathogenic bacteria to antibiotics is one of the treatment problems all over the world. Currently, reports show that the level of resistance in UPEC bacteria is escalating. This issue is especially noticeable in countries where antibiotics are consumed inappropriately and indiscriminately. Determining the pattern of antibiotic resistance in common pathogenic bacteria is important to guide experimental and specific treatments against specific pathogens. Urinary tract infection is one of the most common infections among hospitalized patients and laboratory referees. Moreover, its frequency is classified after respiratory infection. The urinary system's susceptibility to infection, along with its potential for serious complications such as kidney failure, blood infection, and premature birth indicate the importance of diagnosing and treating this disease. Urinary tract infections (UTIs) are among the most common bacterial infections caused by the growth and colonization of pathogens in the urinary tract, which affect millions of people worldwide every year and cause a significant economic burden on the government. Due to the anatomical differences, the prevalence of this infection is much higher in women than in men, so more than half of the women suffer from UTI infections at least once (1). The epidemiology of UTI infection is not reported in many countries, which is why it is difficult to check its exact prevalence (2). However, due to the spread of resistance to antibiotics and the high level of virulence, there is evidence of scattered epidemics and the spread of the disease. According to the colonization range of UPEC (Uropathogenic *E. coli*) strains, UTI infections are known as cystitis (lower parts such as the bladder) and pyelonephritis (upper parts like the kidney), which are often acquired and related to the catheter. Symptoms and clinical complications of pyelonephritis can range from painful urination in

the urinary tract or uncomplicated cystitis to severe systemic disease with abdominal or back pain, fever, sepsis, as well as bladder or kidney failure. Biological factors (such as age and gender) and behavioral factors of the host (such as sexual activities and the response of the host's immune system) as well as the type of infecting microorganism play an important role in the spread and prevalence of this infection (2, 3). Several reports show that bacteria play an important role in causing UTIs, among which E. coli is one of the most common pathogens associated with UTIs (1, 3). *E. coli* is a gram-negative, motile, rod-shaped bacterium that can grow at different temperatures and tolerate acidic conditions up to pH = 4.4. *E. coli* forms a part of the intestinal flora of humans and other mammals, which can turn into a pathogenic form under certain conditions. Pathogenic strains are also divided into two main categories: enteric *E. coli* (intestinal) and extraintestinal *E. coli* (ExPEC). Enteric pathogenic *E. coli* strains include EPEC, EIEC, ETEC, EHEC, EAEC and DAEC pathotypes, and the most important ExPEC strains are UPEC strains. Reports show that UPEC strains are responsible for about 90% of uncomplicated cystitis and pyelonephritis infections (4, 5). The purpose of this study is to investigate the prevalence of uropathogenic Escherichia coli and virulence factors among uropathogenic Escherichia coli strains and to investigate the antibiotic sensitivity of Escherichia coli isolates isolated from urine samples.

MATERIAL AND METHODS

The objective of this research is to collect, isolate, and identify uropathogenic *E. coli* strains, as well as investigate the antibiotic resistance profile and prevalence of certain virulence genes (*hly, cnf-1, afa, fim, aer, and papC*) in samples obtained from patients suspected of urinary tract infections at hospitals in the city of Dhi Qar, Iraq.

Collection of samples

In this study, 375 clinical samples of male and female patients suspected of urinary tract infection were collected in hospitals in Dhi Qar city, Iraq from 01/06/2019 to 29/12/2019. After collection, the samples were transferred to the laboratory under sterile conditions and kept in the refrigerator.

Preparation of culture medium (NA)

To grow and multiply bacterial strains in this research, NA, NB and EMB media will be utilized. To prepare 100 ml of Nutrient Agar culture medium, 2.8 g of NA powder was added to an Erlenmeyer flask containing 100 ml of deionized water. After brief heating and dissolving the contents, the medium will be autoclaved at 121°C for 20 minutes for sterilization. After passing time, the culture medium was transferred into the plates and stored in the refrigerator.

Table 1. Antibiogram discs used in this research

Antibiotic	Antibiotic class
Amikacin	**AN**
Ciprofloxacin	**CIP**
Amoxicillin	**AMC**
Ceftazidime	**CAZ**
Trimethoprim	**TMP**
Ticarcillin	**TI**
Gentamicin	**GM**
Piperacillin	**PI**
Nitrofurantoin	**NIT**
Norfloxacin	**NOR**
Ceftriaxone	**CTR**
Cefixime	**CFM**
Nalidixic acid	**NA**

Culturing and recovery of clinical samples

In this research, approximately 100 clinical samples were collected from individuals suspected of urinary tract infection. To culture, revive, and purify the mentioned isolates, they were initially cultured on an NB culture medium. Subsequently, for purification and colony examination, they were cultured on an EMB medium. To achieve this, several colonies were aseptically removed from the solid culture using a sterile loop. These colonies were then placed individually in an Erlenmeyer flask containing 10 ml of NB liquid culture medium. The culture plates were incubated at 37ºC for 24 hours. Following the revival and refreshment of the samples, bacterial isolates that demonstrated growth were cultured on an EMB culture medium for purification and morphological examination.

Antibiotic resistance pattern analysis

In this study, the antibiotic discs listed in the table were utilized to evaluate the antibiotic resistance profile, following the protocol provided by the Clinical and Laboratory Standards Organization (CLSI). For this purpose, each of the isolates was cultured in the NB culture medium. Subsequently, from the logarithmic culture of each isolate, a 20 µl bacterial suspension with a concentration of 0.5 McFarland (equivalent to 1.5 x 10^8 CFU/ml) was added to the solid culture medium of Muller Hinton. Once inoculated, the bacterial solution was completely spread on the surface of the culture medium using a sterile swab. Finally, each of the antibiogram discs was removed using sterile tweezers and placed on the plate at specific intervals. After closing the lid of the plate, all the plates were incubated for 24 hours at 37°C. Finally, the diameter of the growth halo was measured, and the results were reported as resistant, semi-sensitive, or sensitive.

Genomic DNA extraction

To investigate the distribution of pathogenic genes among uropathogenic E. coli strains, the polymerase chain reaction (PCR) method was employed. Initially, the DNA genome of each isolate was extracted using the boiling method. To accomplish this, overnight cultures of each bacterial isolate were grown in a nutrient broth culture medium under optimal conditions. Subsequently, a specific volume of the bacterial suspension was inoculated into 1.5 ml vials. After centrifugation at 12,000 rpm for 5 minutes and washing the cell sediment with deionized water, 250 µl of distilled water was added to each vial. The samples were then subjected to vortexing, followed by placement in a bain-marie set at 100 ºC for 15 minutes. This step aimed to induce a temperature shock and disrupt the cell wall. Subsequently, the microtubes were transferred to a freezer set at -20 ºC for 1 hour to further enhance cell wall destruction. After the designated time had elapsed, the vials were removed from the freezer and subjected to centrifugation at 12,000 rpm for 10 minutes. This step facilitated the separation of genomic DNA from bacterial debris. Finally, the supernatant containing the extracted genomic DNA was carefully transferred to new sterile microtubes and utilized as a template for PCR analysis.

Agarose gel electrophoresis

Horizontal agarose gel electrophoresis was employed to analyze the amplicons generated by the PCR reaction and assess the presence of bands corresponding to the targeted pathogenic genes.

RESULTS

Figure 1 shows the isolates grown on an EMB medium. In this environment, *E. coli* isolates by fermenting the lactose in the culture medium lead to the production of stable acetaldehyde and compounds of eosin Y and methylene blue, and the oxidation of

Fig1. Cultivation of E. coli isolates on EMB medium

these compounds in the vicinity of oxygen causes the appearance of dark-colored colonies with a shiny metallic lustre. Gram-positive bacteria were not able to grow and multiply on the culture medium due to the presence of eosin and methylene blue. The results showed that among the mentioned isolates, 100% of the samples can grow and survive on an EMB medium.

Figure 2 shows the distribution of uropathogens isolated by age group. Isolates were most frequent among patients aged 41-50 years. Over 58% of isolates came from patients between 30 and 60 years old.

In this research, the agar diffusion method (disk diffusion) was used to check the antibiotic sensitivity of UPEC isolates (Figure 3). The diameter of the non-growth halo around each antibiotic disk was measured and interpreted as resistant (R), intermediate (I), or susceptible (S) according to CLSI standards in Figure (5-4).

As can be seen in Figure 4, the highest resistance and sensitivity among UPEC isolates belong to amoxicillin (82.37%) and amikacin (92.35%) antibiotics, respectively. The resistance rates to ticarcillin, piperacillin, trimethoprim, nalidixic acid, cefixime, ceftriaxone, ceftazidime, norfloxacin, nitrofurantoin, gentamicin, ciprofloxacin, and amikacin were determined to be 81.37%, 80.39%, 78.43%, 74.0%, 55.92%, 62.74%, 55.88%, 48.03%, 40.19%, 27.45%, 28.41%, 36.27%, and 8.65%, respectively.

Research indicates that Enterobacteriaceae bacteria are usually resistant to antibiotics, and this resistance is caused by multiple inherited and acquired mechanisms. Beta-lactam antibiotics are often prescribed to treat Escherichia coli infections, but nowadays the emergence and spread of beta-lactamase-producing strains has also limited the effect of this antibiotic family. Figure 5 shows the frequency of each virulence gene based on gender. The prevalence of *fim, aer, papC, cnf-1, hly and afa* genes among the isolates isolated from male individuals was 38%, 19%, 11%, 6%, 6% and 3%, respectively. Among isolates isolated from female patients with UTI, the *fim* gene (60%) showed the highest prevalence. *aer, papC, cnf-1, hly* and *afa* genes were also in the next ranks with 33%, 19%, 12%, 8% and 7%, respectively. In general, the distribution of

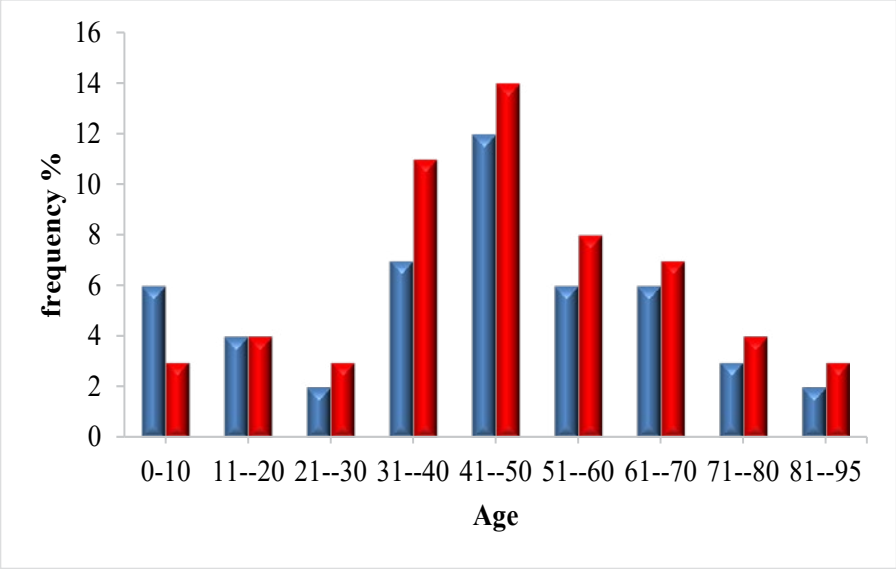

Fig 2. Frequency of isolates isolated from people with UTI according to age range

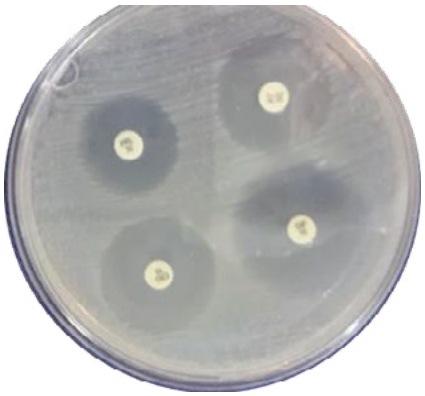

Fig3. Antibiotic sensitivity test results by disk diffusion method

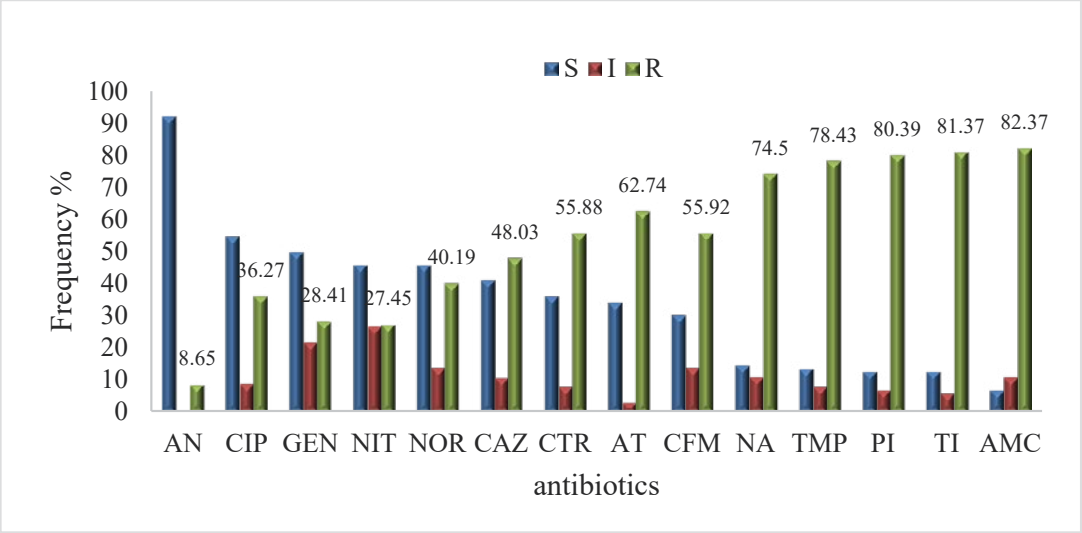

Fig4. Frequency of antibiotic sensitivity and resistance of UPEC isolates by disk diffusion method

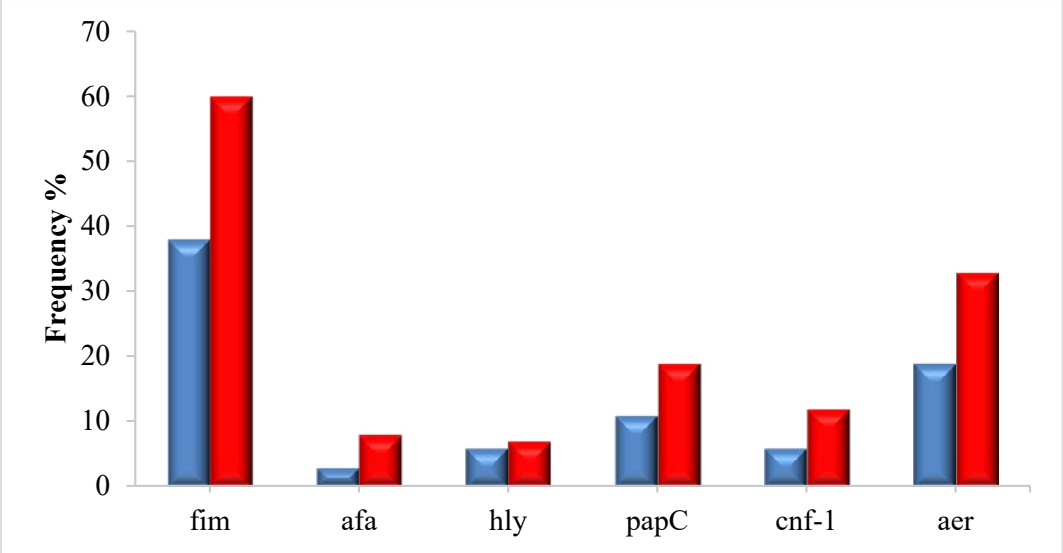

Fig 5. Prevalence of UPEC virulence genes isolated from male and female individuals

virulence genes was observed among female subjects more than male subjects.

Figure 6 shows the products from the amplification of *afa* and *cnf-1* genes. As can be seen, the *afa* gene with a size of 750 bp appeared in more than 11% of the isolates. *cnf-1* gene with a size of 498 bp was expressed in more than 18% of UPEC isolates. Simultaneous expression of these two genes was observed in 3% of isolates (Ec-12, Ec-22, Ec-66).

The *cnf-1* gene is one of the virulence factors in *E. coli* strains, which is located on the bacterial chromosome and is often observed with *hly* genes and S and P fimbriae. This toxin is activated by Rho GTPases RhoA, Rac1 and Cdc42. CNF1 is a single-chain, 115 kDa AB toxin that deaminates the glutamine unit of the Rho family of GTPases. Reports indicate that CNF1 synthesis leads to long-term survival of UPEC in association with human neutrophils (Davis et al., 2005). Figure 7 shows the bands related to *hly* and *cnf-1* genes. The *hly* gene with a size of 1177 bp was present in 13% of UPEC isolates. The distribution of the mentioned gene among men and women was determined as 7% and 6%, respectively. Simultaneous expression of these two genes (*hly* and *cnf-1*) was observed in isolates Ec-2, Ec-11 and Ec-41.Figure 8 shows the bands related to *papC* and *fim* genes. The size of *papC* gene is about 200 bp. In 28% of the mentioned isolates, the above genes were present simultaneously.

Uropathogenic *E. coli* (UPEC) strains wield an arsenal of virulence factors, enabling them to establish a foothold within the urinary tract. These factors orchestrate a coordinated attack, facilitating accumulation, colonization, and immune system evasion through adherence to host epithelial cells.

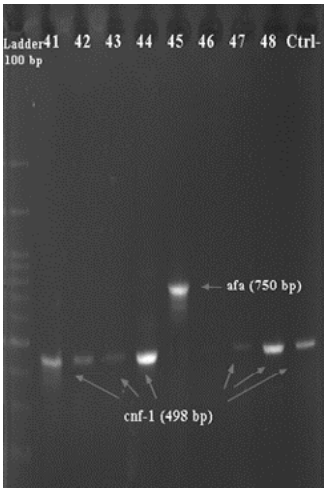

Fig 6. Electrophoresis pattern of PCR products for afa and cnf-1 genes

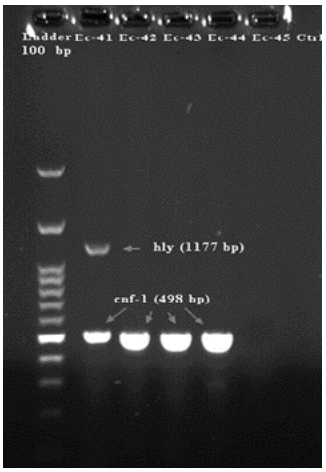

Fig 7. Electrophoresis pattern of PCR products for cnf-1 and hly genes

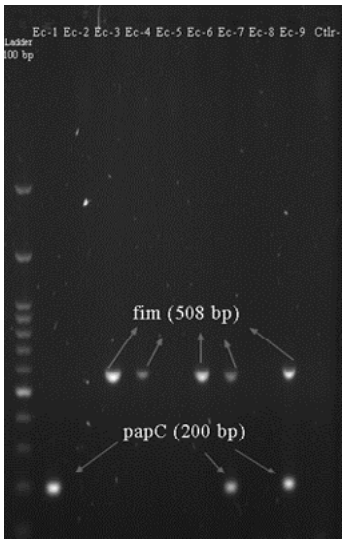

Fig8. Electrophoresis pattern of PCR products for fim and papC genes

Notably, fimbriae, key players in this pathogenic strategy, mediate adhesion to the urinary tract epithelium. Type 1 fimbriae (*fimH*) and P pili (*papC*) represent particularly potent adhesins within this class. This study identified a remarkably high prevalence (approximately 98%) of the *fimH* gene among the examined UPEC strains.

DISCUSSION

Since the discovery of bacteria, humans have continually sought effective drugs to combat the infections they cause, while bacteria have developed mechanisms to destroy antibiotics. The emergence of drug resistance among pathogenic bacteria has presented numerous challenges in treating infectious diseases. Reports of the widespread prevalence of antibiotic-resistant organisms in various hospital departments are now commonplace. Escherichia coli is a common bacterial agent found in human infections, particularly among hospitalized patients and is a major cause of urinary infections. Antimicrobial resistance in Escherichia coli has raised concerns globally due to the increasing rate of resistance. Studies have been conducted to differentiate strains by gender, with Amini and colleagues identifying UPEC strains from clinical samples, revealing that 69.98% of the total 122 samples collected from patients were gender-specific, with 30.33% being women and men (6). The average age of the patients was 47 ± 3.2, and E. coli bacteria were isolated from 36 infected individuals. The isolates were identified through various tests, yielding results consistent with previous findings. By isolating 450 urine samples from men and women suffering from UTI in Iraq (7), they determined that 392 isolates were female and 58 were male. The age range of affected people was also reported between 15 and 75 years. The majority of isolates were from married women (67 cases), unmarried girls (25 cases) and 15-year-old children (11 cases) for both sexes. In the report, out of a total of 364 strains isolated from different hospitals in Iraqi Kurdistan (8), about 81% were isolated from women and 19% from men. The results of the identification of the isolates also showed that more than 41.2% of the isolates (150 samples) belong to the uropathogenic Escherichia coli species (9). In Iraq, various studies have been conducted to evaluate the antibiotic resistance profile among Escherichia coli strains. In one of these researches, by examining the prevalence and pattern of antibiotic sensitivity among uropathogenic E. coli strains among people with urinary tract infection in Zakho, Iraq, they showed that out of 1120 urine samples, only 9.4% of the isolates belonged to UPEC species. The rate of resistance to ceftriaxone and ceftazidime antibiotics was determined to be 52%, which was within the range of our findings. However, the level of resistance to other antibiotics was inconsistent with the data of this research. In the study in the city of Najaf, Iraq, the results of antibiotic resistance profiles among UPEC strains were as follows: CAZ (9.76%), CIP (7.52%),

AN (5.50%), CTR (3.48%), GM (8.42%), NIT (5.17%), the prevalence of some of which (CIP, CTR, NIT) was almost similar to the results of the present study, and the percentage of isolates resistant to CAZ, AN And GM exceeded our findings (10). However, the level of resistance to other antibiotics was inconsistent with the data of this research. In the city of Zakho, Iraq, by examining 400 clinical samples, they showed that 25.35% of the isolates belong to UPEC species. The results of the antibiotic sensitivity pattern among the mentioned isolates showed that the percentage of resistance to amoxicillin, cefixime, ceftriaxone, amikacin, gentamicin, nalidixic acid, norfloxacin, ciprofloxacin and trimethoprim was 6.93%, 1.83%, 2.87%, 2.87%, 8.63%, 4.79%, 9.43%, 6.49% and 7.61%, which is the resistance of isolates to NOR, CIP, NA And TMP is almost consistent with the results of this research.

CONCLUSION

Considering the prevalence of antibiotic resistance of *E. coli* strains in different societies and the role of virulence genes in the occurrence of bacterial infections, choosing the appropriate treatment method and the type of antibiotic used can be particularly important in reducing the prevalence of antibiotic resistance. Afa-positive strains play an important role in the pathogenesis of urinary tract infection (UTI), especially in pregnant women, children and patients with frequent urinary tract infections. The present study showed that the prevalence of virulence genes, *fimH, aer* among uropathogenic Escherichia coli strains isolated from hospitalized patients in this region is high. Therefore, according to the gender, age and type of bacterial infection, determining the pattern of antibiotic resistance profile, screening of pathogenic bacteria, as well as the relationship of virulence genes with the rate of infection in people with UTI periodically can be of great help in improving the treatment conditions and epidemiology studies. Therefore, the above genes can be further studied as targets in therapeutic interventions.

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest.

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Targeting Key Genes in the Early Diagnosis and Treatment of Lung Cancer with a Focus on Personalized Medicine: a Review Article

Milad Pezeshki [1], Abbas Ardalan [2], Mahdi Nakhaee [3], Saeid Ziaei [4*], Roja Valipoor [5], Elahe Tamjidi [6], Rozhin Naseri [7]

[1]MSc in Genetics, Development and Research Group of LifeandMe Company, Tehran, Iran.
[2]Science Based Amitis Gen Tech Dev Group, Tehran, Iran.
[3]Expert in Laboratory Sciences, Development and Research Group of LifeandMe Company, Tehran, Iran.
[4]PhD of Applied Proteomics, Development and Research Group of LifeandMe Company, Tehran, Iran.
[5] Department of Laboratory Medicine, Faculty of Paramedical Sciences, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.
[6]MSc in Physiology Education and Sport Sciences, Shahid Chamran University, Ahvaz, Iran.
[7]MSc Student in Cell and Molecular, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.

**Corresponding author: Saeid Ziaei, Development and Research Group of LifeandMe Company, Tehran, Iran. Email: lifeandme.research@gmail.com*

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Abstract:
IntroductionLung cancer is the prevailing form of cancer globally, with a significant fatality rate among both males and females. Lung cancer is the third most frequent type of cancer in Iran, and it is becoming more common all the time. Patients are frequently diagnosed in the advanced stages of the disease, which contributes to the high death rate. Therefore, the ability to identify molecular markers is essential for both early diagnosis and the choice of conventional treatment for lung cancer. Numerous genetic variations have been found to be strongly linked to the development of lung cancer, according to studies. The aim of this work is to look into the genes that contribute to the development of lung cancer.
Materials and methods: The present review was authored using search terms related to lung cancer, key genes, clinical biomarkers, and early diagnosis that were found on PubMed, NCBI, Scopus, Science Direct, and Google Scholar.
Findings: Since the *EGFR, KRAS, BRAF*, and *TP53* genes are the most significant and involved in the development of lung cancer, finding mutations in these genes can be a valuable clinical diagnostic for lung cancer diagnosis and therapy.
Discussion and conclusion: With an emphasis on personalized medicine, the identification of genes linked to lung cancer may be utilized as clinical biomarkers for the disease's early diagnosis and effective treatment. The state of targeted lung cancer therapy and early detection techniques may be enhanced by molecular biomarkers. In the field of personalized medicine, identifying the genes linked to lung cancer as clinical biomarkers for early diagnosis and assessing treatment response to select a targeted treatment can be crucial in streamlining the therapeutic process, improving treatment response, lowering mortality, and lessening the material and spiritual harm this illness causes.

INTRODUCTION

Cancer is known as one of the main causes of death in economically developed and developing countries. About 12.7 million new cancer cases and 6.7 million cancer deaths occur annually (1). Most of the factors that cause cancer are among the factors that lead to DNA sequence changes or mutations (2). Lung cancer or lung carcinoma is a malignant lung tumor that can be identified by uncontrolled cell growth in lung tissues (3, 4). The incidence and mortality rate of lung cancer has increased significantly around the world (5). Lung cancer is a complex disease caused by

genetic and environmental factors derived from the interaction between these two factors. Lung cancer is the main cause of death due to cancer worldwide so that approximately 1.6 million people die from lung cancer every year (4, 6, 7). Studies have shown that about 8% of lung cancer cases are due to hereditary factors (8). Lung cancer is the second most common cancer in the Western Hemisphere (9). The highest incidence of lung cancer is in North America, Europe and East Asia. The incidence rate of this cancer is lower in Africa and South Asia (4). Lung cancer mortality is higher in developed countries than in less developed countries and is higher in men than women (9). In Iran, lung cancer ranks 7th in men and 10th in women, and it is the second and third leading cause of cancer death among men and women, respectively (10). The incidence of this cancer in Iran is increasing day by day (11). It has been estimated that more than 13% of newly diagnosed cases of cancer and approximately 27% of total cancer deaths are related to lung cancer (12). Despite the diagnostic advances in recent years, most of the cases related to lung cancer are diagnosed in advanced stages, and for this reason, the mortality rate is high (12-14). Lung cancer is of great importance among cancers due to its high prevalence of lethality and low 5-year survival rate (16, 15). Lung cancer is a complex pathological process that is divided into two main groups: small-cell lung cancer and non-small cell lung cancer (18,17). Non-small cell lung cancer is the most common tissue type of lung cancer with a percentage of about 85% (19). Non-small cell lung cancer includes three subgroups: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (4). Lung adenocarcinoma, which occurs on the surface of the lung tissue (4), accounts for most cases of lung cancer and its incidence rate is increasing (20). Several risk factors are involved in the occurrence of lung cancer. Among the known risk factors related to the increased risk of lung cancer, smoking has the strongest association, so it is reported in 85% of lung cancer cases (8). Cigarette smoke contains at least 73 known carcinogens, including benzopyrene. Cigarette smoke inhalation is one of the risk factors for this cancer in non-smokers. Such people usually live or work next to smokers (4). Another risk factor for lung cancer is radon gas. Radon gas is a colorless and odorless gas that is produced through the decay of the radioactive element radium and can cause lung cancer by causing mutations in the genome (21). Radon is the second most common cause of lung cancer in America, which is the cause of death of 21,000 people every year (4). Air pollution caused by burning wood, charcoal or other agricultural products for cooking and heat production is another factor of environmental risk (22). Women who are exposed to coal smoke have twice the risk of contracting it (23). Asbestos is another risk factor that can increase the risk of lung cancer. Smoking and being exposed to asbestos have an aggravating effect on the occurrence of lung cancer. In smokers who work with asbestos, the risk of developing lung cancer is 45 times that of the general population. Genetic factors and age also play an important role in the occurrence of lung cancer. Increasing age increases the risk of lung cancer by increasing the number of mutations in the genome. About 8% of lung cancers are hereditary. In general, the risk of lung cancer increases more than 2 times in people who have first-degree relatives with the disease (4, 24). Other environmental factors include metals such as Aluminium and Aluminium products, cadmium and cadmium compounds, chromium, beryllium and beryllium compounds, iron, steel, nickel-containing compounds, arsenic, mineral arsenic compounds, hematite extracted from underground sources, and combustion products. including the incomplete combustion of coal and the gases resulting from its burning, the smoke resulting from the burning of vehicle fuel, ionizing radiation including X-rays, gamma and plutonium, toxic gases such as methyl ether, bis ether, sulfur-containing gases, and crystalline silica dust (25). Among the risk factors, the genetic factor is considered as the main factor. This study aims to explore the significance of important genes in the development of lung cancer. Determining the genes linked to the development of lung cancer can be crucial to comprehending the lung's carcinogenesis process, facilitating early detection, and enhancing patient care. Lung cancer develops similarly to other cancers in terms of how it spreads. Put another way, the activation of oncogenes or the inactivation of tumor suppressor genes is the initial step towards lung cancer. (4). Changes in these two categories of genes along with changes in DNA repair genes such as the occurrence of mutations and single nucleotide polymorphisms (26) along with other changes in the genome such as deletions, integrations, chromosomal translocations, inversions and epigenetic changes such as changes in DNA methylation. Changes in the tail region of histones or changes in the regulation of microRNAs, all by affecting the inactivation of tumor suppressor genes and the activation of proto-oncogenes, can ultimately lead to the occurrence of lung cancer (4). Therefore, investigating and identifying the genes involved in lung cancer can play a major role in identifying the carcinogenesis process of this disease (12, 27, 28, 29). The most common genetic changes in the genome that can affect the incidence of lung cancer are single nucleotide polymorphisms at the genome level. Polymorphisms are naturally present in human genomic DNA and are used to identify individual differences related to

susceptibility to diseases (30). The presence of these polymorphisms in proto-oncogenes, tumor suppressor genes and DNA repair genes respectively lead to the creation of oncogenes (increased expression of a gene), the lack of function of the protein resulting from tumor suppressor genes and the loss or reduction of repair capacity in DNA repair genes. It leads to lung cancer (7, 15, 27). Therefore, the presence of this polymorphism in the mentioned genes can affect the risk of lung cancer (31). Recent studies to investigate the variants of the genomic range related to lung cancer significantly show that polymorphism changes in the regions of 15q25.1 (CHRNA5/CHRNA3/CHRNB4), 5p15.33 (TERT/CLPTM1L), 6p21.33 (BAT3/MSH5), 22q12 (CHEK2), 15q15.2 (TP53BP1) and 12p13 (RAD52) are effective in the risk of lung cancer (31-33). There are other susceptible regions in chromosomal positions 13q12.12, 3q28, 6q23-25, 18p11.23, 2p22.2, 14q13.1, 16p13, 20q13.11, 1q42-43, 7p12.1-12.3, and 7q31.3. Various studies have shown a significant association with the risk of lung cancer (12, 34-36). The mentioned regions related to lung cancer contain genes that are in three general categories proto-oncogenes, tumor suppressor genes and repair genes (2, 4). The presence of a polymorphism on a proto-oncogene can lead to an increase in product production and its transformation into an oncogene and is related to the risk of lung cancer (8). The presence of polymorphisms in tumor suppressor genes can lead to the production of inefficient proteins and is associated with the risk of lung cancer (37). On the other hand, the placement of polymorphism on DNA repair genes can also lead to a change (decrease) in DNA repair capacity and is associated with the risk of lung cancer (38). Mutations in two groups of proto-oncogene genes and tumor suppressor genes are the starting point of the carcinogenic process in the lung. The occurrence of mutation in proto-oncogenes causes excessive production of the product of a protoncogene and turns it into a cancerous gene that causes cancer. The occurrence of mutation in tumor suppressor genes causes the loss of function of these genes and can lead to the occurrence of lung cancer (2, 4, 28). Various studies have been conducted, and about 50 tumor suppressor genes and 100 oncogenes that play a role in the cell cycle and the incidence of lung cancer have been identified and investigated (39). Identification of mutations in genes that have the greatest effect on the occurrence of lung cancer can play an important role in identifying biomarkers for early diagnosis, reducing the rate of this cancer and also reducing material and spiritual damages (40, 41). Mutations in cancer genes and tumor suppressor genes are necessary to cause lung cancer. Identification of mutations in these two groups of genes can play a major role in lung cancer screening (prognosis and prediction) as molecular markers.

MATERIALS AND METHODS

In this research, to write this review article, the keywords of lung cancer, clinical biomarkers, key genes, and early diagnosis were searched in PubMed, NCBI, Scopus, Science Direct and Google Scholar websites, and after extracting related sources, the present article was based on They were written. Our goal in this study is to investigate the genes involved in the occurrence of lung cancer in such a way that after identifying and listing the genes involved in the occurrence of lung cancer, we investigated the genes that have the highest mutation rate and then the names of the common mutations. We examine the damaging process of mutations and its importance in the field of early diagnosis and treatment of lung cancer.

FINDINGS

Several oncogenes and tumor suppressor genes have been identified whose mutations are associated with lung cancer (42). *KRAS* gene, which is located at chromosomal position 12p12.1, is a member of the RAS family and one of the first oncogenes involved in lung cancer. Activating mutations in the *KRAS* oncogene are the most common oncogenic changes in lung adenocarcinoma, occurring in up to 40% of cases (42, 43). The second most important gene in the occurrence of lung cancer is the *EGFR* oncogene, which is located at chromosomal position 7p12.1-12.3 (44). Mutations in this gene lead to lung cancer by increasing its expression. Increased expression of this gene due to mutation has been observed in 43-89% of lung cancer cases. More than 90% of point mutations and deletions occur in exon 19 of this gene (42). The most important tumor suppressor gene involved in the occurrence of lung cancer is *TP53*, which is located in the chromosomal position 17p13 and plays an important role in preventing the occurrence of lung cancer. Mutations in this gene can cause lung cancer, as mutations in this gene have been reported in 50-60% of lung cancer cases (42, 45). Among other genes involved in the occurrence of lung cancer is the *BRAF* oncogene at chromosomal position 7q34, which is a member of the RAS family, and mutations in this gene have been reported in up to 8% of lung cancer cases. The hot spot of this gene for the occurrence of mutation is codon 600 of this gene (42, 46). Among other genes involved in the occurrence of lung cancer, we can mention the *HER2* gene, the mutations in exon 20 of this gene have been reported in about 2 to 4 percent of lung cancer cases. *EML4* and *ALK* genes are among the other effective genes in the occurrence of lung cancer, and changes in these genes have been reported in about 3 to 7 percent of lung cancer cases. Proto-oncogene ROS1, which is located at chromosomal location 6p22, is associated with lung

Table1. Names and frequencies of genes involved in the occurrence of lung cancer (2).

Gene	Cancer type	Genetic Alteration	Frequency%
EGFR	Adenocarcinoma	Point mutations and copy number variants	30-40
KRAS	Adenocarcinoma	Point mutations	20-30
MET	Adenocarcinoma	Slippage mutations, increased expression	2-5
ALK	Adenocarcinoma	Integration	3-7
BRAF	Adenocarcinoma	Point mutations	۰.05-5
ROS1	Adenocarcinoma	Integration	2-3
RET	Adenocarcinoma	Gene rearrangement, integration and point mutations	1-2
NTRK	Adenocarcinoma	& integration Gene rearrangement	1-2
HER2	Adenocarcinoma	Introgression, point mutations and multiplication	1-5
PTEN	Adenocarcinoma	Copy number variant	1.7
PDGFRA	Adenocarcinoma	Copy number variant	6-7
PIK3CA	Adenocarcinoma	Copy number variant	5
TP53	Adenocarcinoma	Copy number variant	52
ERBB2	Adenocarcinoma	Copy number variant	2-5
TERT	Adenocarcinoma	Copy number variant	7.5
CDKN2A	Adenocarcinoma	Copy number variant	7
FGFR	Squamous cell carcinoma	Integration & Point mutation	23
TP53	Squamous cell carcinoma	Point mutation	79
NF1	Squamous cell carcinoma	Point mutation	10
DDR2	Squamous cell carcinoma	Point mutation	2-3
PDGFRA	Squamous cell carcinoma	Gene multiplication	4
PIK3CA	Squamous cell carcinoma	Gene multiplication	15
PTEN	Squamous cell carcinoma	Point mutation	10
SOX2	Squamous cell carcinoma	Copy number variant	8
CDKN2A	Squamous cell carcinoma	Gene multiplication & copy number variant	15

cancer in case of mutation. *RET* gene is a new oncogene located in chromosomal location 10q11.2 and has been reported in about 1.3% of lung cancer cases (42). Table 1 lists the names of oncogenes involved in the occurrence of lung cancer along with their frequency. Among the mutations in the genes involved in the occurrence of lung cancer, *EGFR*, *KRAS* and *BRAF* oncogenes along with the tumor suppressor gene *TP53* have the highest importance and role in the occurrence of lung cancer (42). Thus, identifying the mutations in all of this genes that most influence the development of lung cancer can be crucial to comprehending the pathophysiological mechanism of lung cancer. (40, 41).

EGFR gene mutations and the incidence of lung cancer

Epidermal growth factor receptor (EGFR) is a membrane glycoprotein that has tyrosine kinase activity (44) and is a member of the ErbB family. Other members of this family include ErbB2, ErbB3 and ErbB4 (47). EGFR plays an important role in regulating and controlling many different signaling pathways such as growth, cell proliferation, cell adhesion, differentiation, migration and survival (44-48). The epidermal growth factor receptor is coded by the *EGFR* gene, which is located at chromosomal location 7p12.1-12.3. This gene consists of 28 exons (44). EGFR activation as a result of epidermal growth factor binding leads to the activation of intracellular signaling cascades, which lead to the regulation and control of normal cell processes (47). The *EGFR* gene is the most important gene that plays a significant role in the risk of lung cancer at the genome level, in such a way that the presence of mutations in this gene leads to an increase in its expression and causes the conversion of the *EGFR* proto-oncogene into the *EGFR* oncogene, which as a result causes carcinogenesis in Different cells of the lung tissue. Therefore, by discovering the changes created, it is possible to predict and manage the process of carcinogenesis in the lung (20, 49, 50). According to Figure (1), binding of epidermal growth factor receptor to its ligand causes autophosphorylation through the activity of tyrosine kinase located in the second intracellular region and triggers several signal transmission cascades (51). *EGFR* signals activate at least two intracellular pathways in parallel. One of these pathways is the MAP kinase (MAPK) pathway, which regulates the G1 checkpoint in the cell cycle. When *EGFR* is activated, the MAPK pathway sends a signal to the nucleus through the active forms of *RAS*, *RAF* and *MEK* gene and causes cell proliferation (20). The presence of mutation on this gene increases its expression and leads to an increase in the signals sent to the nucleus as a result of the cell going out of normal reproduction and leading to the occurrence of cancer in different lung tissue cells (49). Mutations in the *EGFR* gene are often located on exons 18, 19, 20, and 21 (52). Among these, the most changes include deletions in exon number 19 and point mutation in exon number 21 of this gene (53). Two mutations, L858R and del exon 19, located on exons 21 and 19, respectively, have been reported in more than 90% of lung cancer

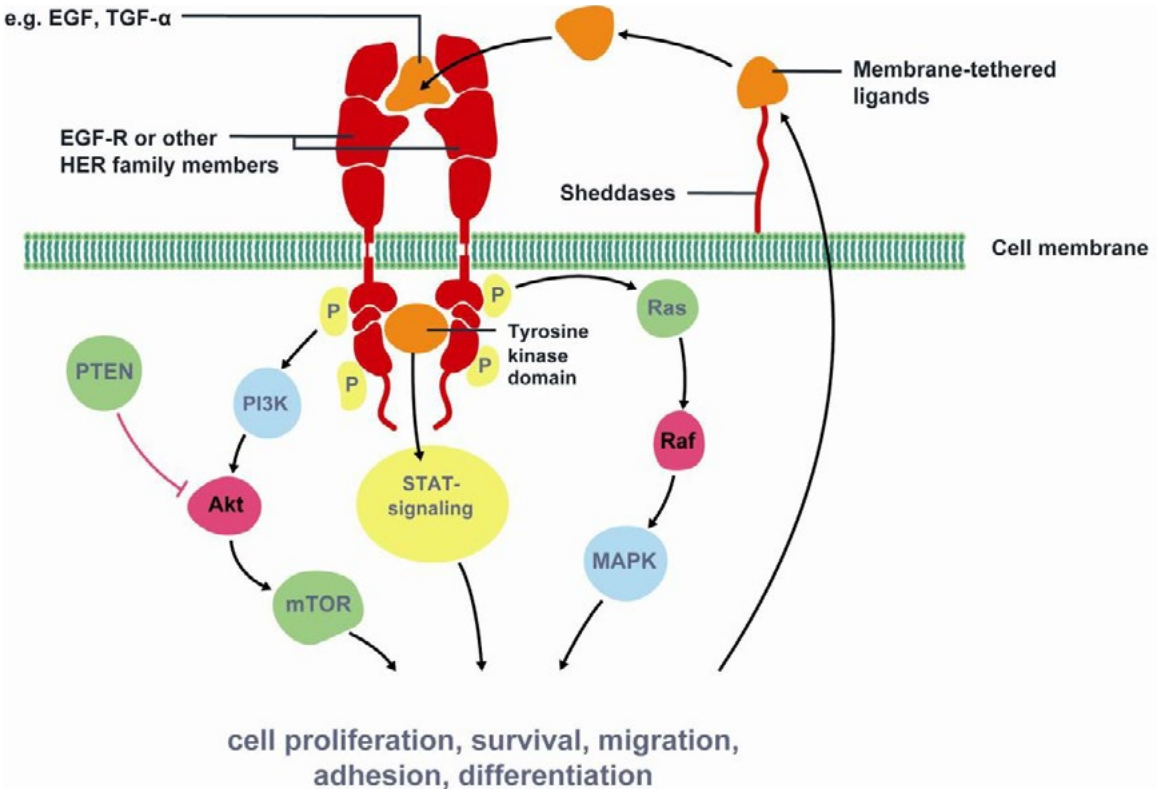

Fig 1. The role of the epidermal growth factor receptor (*EGFR*) gene pathway in the occurrence of lung cancer.

cases (54). The names of the mutations on exon 18 to 21 that play a role in the occurrence of lung cancer are given in Table 2.

These mutations in the *EGFR* gene are important from the point of view that they can be used in the field of diagnosis and treatment. Choosing a suitable treatment based on the molecular profile in the treatment of lung cancer in the field of personal medicine can play an important role in increasing the response to treatment, increasing the survival rate of patients, and reducing the mortality rate and material and spiritual damages. Identification and diagnosis of *EGFR* gene mutations and a correct understanding of the use of anti-*EGFR* tyrosine kinase inhibitors and the targeted use of common treatments (chemotherapy and radiation therapy) can play an important role in increasing the response rate to treatment, increasing patient survival, and reducing the death rate. and material and spiritual damages. Therefore, oncologists consider *EGFR* gene mutations to evaluate resistance to chemotherapy and radiation therapy and, if necessary, to use anti-*EGFR* tyrosine kinase inhibitors. The use of anti-*EGFR* tyrosine kinase inhibitors in lung cancer patients with *EGFR* mutations such as L858R and L861Q reduces the activity of tyrosine kinase and then prevents cancer progression (28, 55).

KRAS gene mutations and the incidence of lung cancer

KRAS gene, which is located at chromosomal position 12p12.1, is a member of the *RAS* family and one of the most important oncogenes involved in lung cancer. Activating mutations in the *KRAS* oncogene are the most common oncogenic changes in lung adenocarcinoma, which occur in up to 40% of cases (56, 57). According to Figure 2, KRAS is one of the G-proteins of the RAS family with GTPase activity, which is located in the inner space of the plasma membrane. Its function is that it acts as a double molecular switch. When it is bound to GTP, it is in the active state and when it is bound to GDP, it is in the inactive state. When it is active, the RAS-GTP complex activates several signaling cascades, such as the Raf-MEK-ERK, PI3K-AKT-mTOR and RalGDS-RalA/B pathways, as well as the TIAM1-RAC1 pathway. All these pathways play pivotal roles in cell proliferation, apoptosis, survival and growth (57). Activation of *KRAS* mutations prevents it from being hydrolyzed, so it remains permanently "on" and leads to continuous activity of downstream receptors (58). In other words, the mutation in the *KRAS* oncogene leads to a continuous message from the outside to the nucleus and then causes unregulated cell proliferation and the occurrence of cancer in lung tissue cells (59). Most mutations of this gene in cases of lung cancer occur on exon 2 and codons 12 and 13 of this gene (59, 60). These mutations are more than 95% in codon 12 and more than 80% in codon 13 of this gene. The most common mutated codon reported in lung cancer patients is the *KRAS -G12C* mutation, which has a frequency of about 40%. Other common mutations of this gene are G12V

Table2. Names of *EGFR* gene mutations involved in lung cancer

Mutation number	Exon	Nucleotide change	Amino acid change
1	18	c.2155G>A	p.G719S
2	18	c.2155G>T	p.G719C
3	18	c.2156G>A	p.G719D
4	18	c.2156G>C	p.G719A
5	19	c.2235_2249 del 15	Glu746_Ala750del
6	19	c.2235_2252> AAT	Glu746_Thr751delinsIle
7	19	c. 2236_2253 del 18	Glu746_Ser752del
8	19	c. 2237_2251 del 15	Glu746_Thr751delinsAla
9	19	c. 2237_2254 del 18	Glu746_Ser752>Ala
10	19	c. 2237_2255>T	Glu746_Ser752delinsVal
11	19	c. 2236_2250 del 15	Glu746_Ala750del
12	19	c.2238_2255 del 18	Glu746_Ser752delinsAsp
13	19	c. 2238_2248 >GC	Leu747_Ala750>Pro
14	19	c. 2238_2252 >GCA	Leu747_Thr751delinsGln
15	19	c. 2239_2247 del 9	Leu747_Glu749del
16	19	c. 2239_2253 del 15	Leu747_Thr751del
17	19	c. 2239_2256 del 18	Leu747_Ser752del
18	19	c. 2239_2258 >CA	Leu747_Pro753delinsGln
19	19	C. 2240_2251 del 12	Leu747_Thr751delinsSer
20	19	c. 2240_2257 del 18	Leu747_Pro753delinsSer
21	19	c. 2240_2254 del 15	Leu747_Thr751del
22	19	c. 2239_2251>C	Leu747_Thr751deinsPro
23	20	c.2369C>T	p.T790M
24	20	c.2303G>T	p.S768I
25	20	c.2307_2308insGCCAGCGTG	p.V769_D770insASV
26	20	c.2310_2311insGGT	p.D770_N771insG
27	20	c.2319_2320insCAC	p.H773_V774insH
28	21	c. 2573 T>G	Leu858Arg
29	21	c. 2582 T>A	Leu861Gln

and G12D with 21 and 18 percent respectively (61). The most important mutations of codons 12 and 13 of this gene are given in Table (3) (62). Identification of *KRAS* gene mutations can be used in the field of early diagnosis in the field of therapy using anti-*KRAS* tyrosine kinase inhibitors (28).

TP53 gene mutations and the occurrence of lung cancer

The most important tumor suppressor gene involved in the occurrence of lung cancer is TP53, which is located in the chromosomal position 17p13 and consists of 19,149 base pairs and 11 exons, which plays an important role in regulating the activity of genes involved in the processes of DNA repair, metabolism, cell cycle arrest, It has apoptosis and senescence, the result of which is the prevention of lung cancer. Mutations in this gene can cause lung cancer, as mutations in this gene have been reported in 50-60% of lung cancer cases (42, 45, 63). In healthy cells, P53 is normally present in a small amount in the cell. When

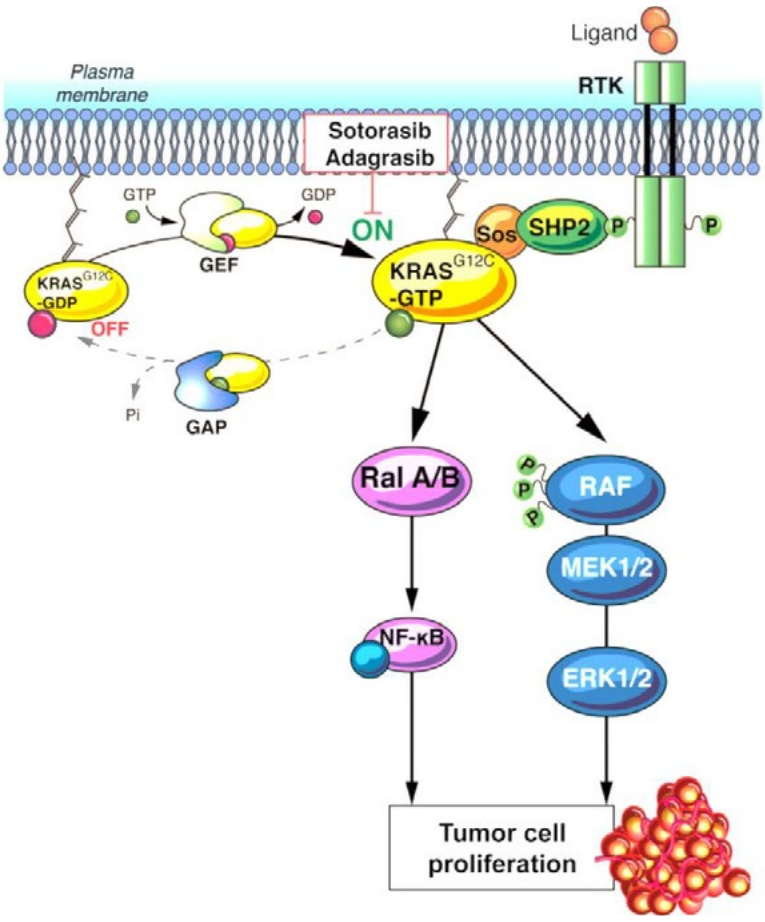

Fig 2. Schematic of molecular mechanism of mutation in *KRAS* gene and how lung cancer occurs .

cells are subjected to hypoxia stress or DNA damage, *P53* prevents its degradation and causes a rapid increase in its concentration inside the cell, so *P53* can bind to the promoter. DNA binds and stimulates the transcription of repair genes. If the damage caused cannot be repaired, then *P53* prevents the occurrence of cancer in lung tissue cells by transcription of genes involved in apoptosis and induction of apoptosis (63). Cells with mutated *P53* protein cannot bind to the DNA promoter sequence, so the damaged cell is not repaired and does not enter the path of apoptosis, and as a result, the path of cancer takes place (Figure 3). Such cells that lose their tumor suppressor gene activity are prone to multiply and become cancerous (63, 64).

More than 90% of the mutations that occur in this gene are located in the second DNA binding region of this gene. Most mutations of this gene in lung cancer are located on exons 5, 7, and 8 and codons 157, 158, 245, 248, and 273 (64, 63, 65). The most important mutations of this gene are given in Table (4). Knowing the mutations of this gene can also be beneficial in the diagnosis and treatment of patients. Several studies have shown that the presence of a mutation in the *P53* gene leads to resistance to lung cancer chemotherapy in laboratory and in vivo conditions. Knowing the status of *P53* is important and necessary to use chemotherapy and radiotherapy. Gene therapy is the best treatment solution in these cases (64).

BRAF gene mutations and the incidence of lung cancer

BRAF oncogene located at chromosomal position 7q34 is a member of the RAS family and mutations in this gene have been reported in up to 8% of lung cancer cases (42, 46). The *BRAF* gene is a serine/threonine kinase that controls cell proliferation by interacting with RAS-GTPs and downstream proteins from the MAPK family (28, 66). Mutation in exon 15 of this gene causes oncogenic activity in this gene and causes cancer in lung tissue cells (67). The most important and the only mutation reported in this gene in cases of lung cancer is V600E, which is located on exon number 15 of this gene. According to figure (4), The c.1799T>A (p.V600E) mutation of this gene causes the *BRAF* proto-oncogene to become the *BRAF* oncogene and the continuous activity of this gene, therefore, the natural growth and reproduction mode is out of reach and the cancerous process is started in different lung tissue cells. Therefore, the examination and testing of the *BRAF* gene mutation in lung cancer can be used as a diagnostic marker for early diagnosis. Also, this

Table 3. Names of *KRAS* gene mutations involved in lung cancer

Mutation number	Exon	Nucleotide change	Amino acid change
1	2	c.35G>A	G12D
2	2	c.34G>T	G12C
3	2	c.34G>A	G12S
4	2	c.34G>C	G12R
5	2	c.35G>C	G12A
6	2	c.35G>T	G12V
7	2	c.38G>A	G13D
8	2	c.37G > T	G13C
9	2	c.37G > A	G13S
10	2	c.37G > C	G13R
11	2	c. 175G>A	A59T
12	3	c. 181C>A	Q61K
13	3	c. 182A>T	Q61L
14	4	c. 182A>G	Q61R
15	4	c. 183A>C	Q61H
16	4	c. 183A>T	Q61H
17	4	c.351A>C	K117N
18	4	c. 351A>T	K117N
19	4	c. 436G>A	Al46T
20	4	c. 437C>T	A146V
21	4	c. 436G>C	A146P
22	4	c.35G>A	G12D

mutation can be used as a therapeutic target by using *BRAF* inhibitors to reduce cell proliferation and inhibit lung cancer progression (66- 68).

In this study, the genes involved in the incidence of lung cancer were identified and investigated. As a result of this study, it was determined that *EGFR*, *KRAS* and *BRAF* proto-oncogenes along with the *TP53* tumor suppressor gene showed the highest mutation frequency among all genes involved in lung cancer. The mentioned genes are known as central genes in lung cancer and have different hotspots for mutation. These hotspots in the *EGFR* gene are often located on exons 18, 19, 20 and 21, and among them, two mutations L858R and del exon 19 on exons 21 and 19, respectively, are included in more than 90% of lung cancer cases. In most of the studies conducted on the mentioned *EGFR* gene mutations, it was found that these mutations play a significant role in the field of response to treatment and tyrosine kinase inhibitors. The results of this study showed that the *KRAS* gene is the second most important proto-oncogene involved in the occurrence of lung cancer so most of the mutations of the mentioned gene in cases of lung cancer occur on codons 12 and 13 of exon 2 of this gene. These mutations are more than 95% in codon 12 and more than 80% in codon 13 of this gene. The most common mutation reported in patients is the *KRAS -G12C* mutation, which has a frequency of about 40%. Other common mutations of this gene are *G12V* and *G12D* with 21 and 18 percent respectively.

BRAF gene was identified as the third most important proto-oncogene involved in lung cancer. The

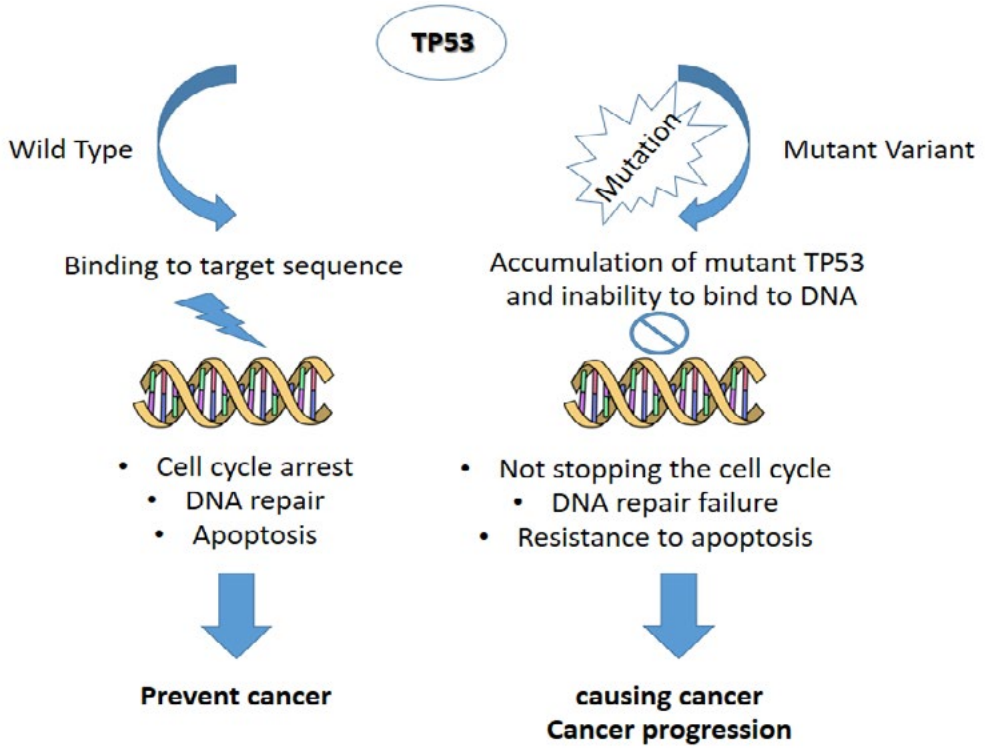

Fig 3. Schematic of how lung cancer occurs due to mutations in the *TP53* gene.

Table 4. Names of *TP53* gene mutations involved in lung cancer

Mutation number	Exon	Nucleotide change	Amino acid change
1	7	c.733G>T	p.G245C
2	7	c.741- 742CC>TT	p.R248W
3	8	c.818G>A	p.R273H
4	5	c.524G>T	p.R175L
5	7	c.743G>A	p.R248Q
6	7	c.747G>T	p.R249S
7	8	c.817C>T	p.R273C
8	5	c.430C>T	p.Q144*
9	5	c.438G>A	p.W146*
10	5	c.440T>A	p.V147D
11	5	c.472C>G	p.R158G
12	5	c.499C>T	p.Q167*
13	5	c.524G>T	p.R175L
14	7	c.722C>T	p.S241F
15	8	c.738G>C	p.M246I
16	8	c.818G>T	p.R273L

only mutation in this gene is c.1799T>A (p.V600E), which causes the BRAF proto-oncogene to become the *BRAF* oncogene, therefore, the natural growth and reproduction mode is out of reach and the cancerous

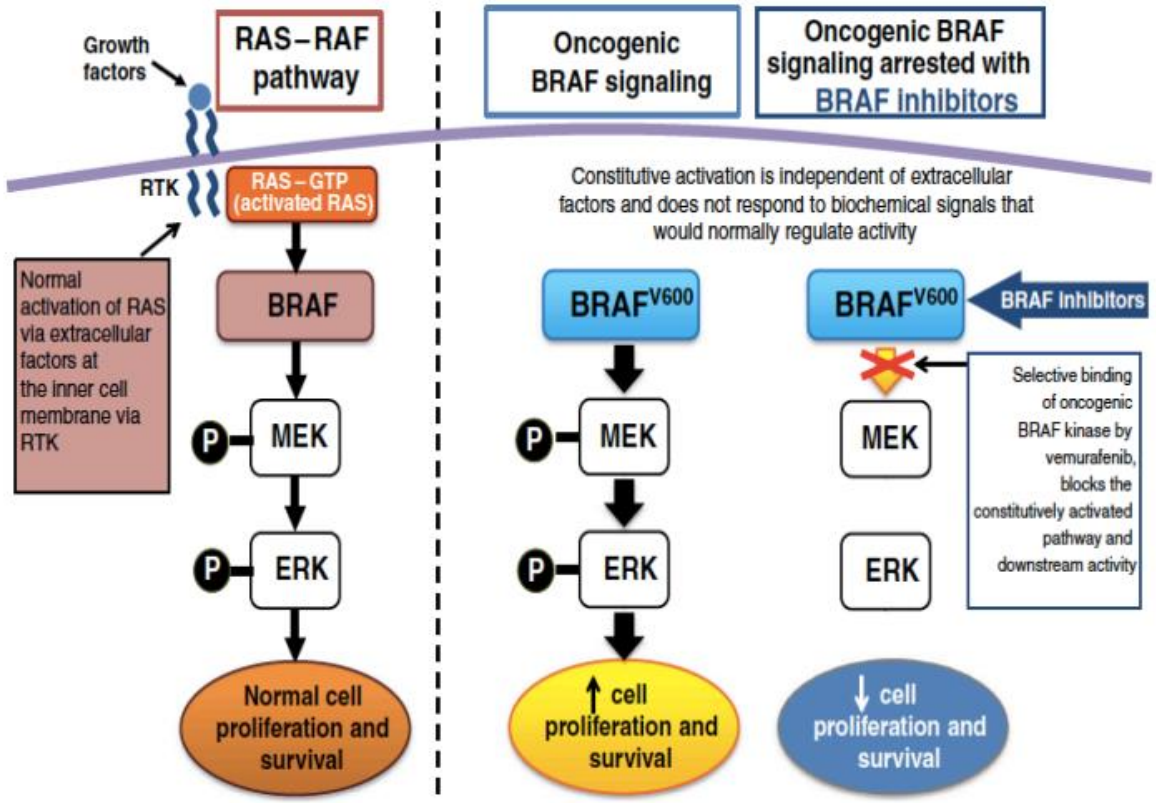

Fig4. Schematic of the molecular mechanism of the mutated *BRAF* gene and the incidence of lung cancer.

process is started in different lung tissue cells. The mentioned mutation test in the BRAF gene in lung cancer can be used as a diagnostic marker for early diagnosis and also as a therapeutic target by using BRAF inhibitors to reduce cell proliferation and inhibit the progression of lung cancer. The fourth gene and the only tumor suppressor gene that has the highest mutation rate in lung cancer is the *TP53* gene. The hotspots of mutation in this gene are exons 5, 7 and 8 and codons 157, 158, 245, 248 and 273. Knowing the mutations of this gene can also be beneficial in the diagnosis and treatment of patients in such a way that the presence of mutations in the *p53* gene leads to resistance to lung cancer chemotherapy in laboratory and in vivo conditions. Knowing the status of p53 is important and necessary to use chemotherapy and radiotherapy. Gene therapy is the best treatment solution in these cases. Examining the mutations of the mentioned genes, which have the highest mutation rate in the pathogenic process of lung cancer, can be used as clinical biomarkers for diagnosis, prognosis and response to treatment related to lung cancer.

DISCUSSION AND CONCLUSION

Several studies have been conducted on the frequency of mutations in the mentioned genes in people with lung cancer. In a study conducted by Li et al. in 2014 in China, the frequency of *EGFR* and *KRAS* gene mutations in lung tumors was studied. The mutation rate in the *EGFR* gene was reported to be 37% and in the *KRAS* gene to be 20%. Examining the mutation in these genes, in addition to predicting the risk of lung cancer, can be useful in the process of choosing a treatment strategy (52). In another study conducted by Wu et al. in 2012 in Taiwan, the status and frequency of *EGFR* gene mutations in patients with lung adenocarcinoma were analyzed using the sequencing method. In this study, about 70% of patients had mutations in the mentioned gene. The analysis of mutations of this gene can play an important role in early diagnosis and prognosis, as well as the selection of treatment methods related to the use of tyrosine kinase inhibitors (54). In a study conducted by Fouad et al. in 2012, the mutations of exon 4, 18, 19, 20 and 21 of the *EGFR* gene were investigated in lung tumor samples using the sequencing method. Mutations in the *EGFR* gene were observed in about 20% of tumor samples. Among the four exon mutations, the L858R mutation in exon 21 and deletion in exon 19 were the most frequent mutations (20).

In a study conducted by Fernando Lopez et al. 2012 in Spain, mutations in the *EGFR* gene were investigated in lung tumor samples. As a result of this study, more than 90% of mutation cases were reported, including deletions in exon 19 and point mutation L858R (77). In a study conducted by Yamamoto et al. 2017 in Japan,

the mutations of *EGFR* and *KRAS* genes in lung tumor samples were investigated using the NGS method. As a result of this study, mutations in *EGFR* gene were reported in about 40% of cases, and Ex19del and L858R mutations were the most frequent. Meanwhile, about 20% of mutation cases were observed in exon 2 of the *KRAS* gene (78). In the study conducted by Jing et al. in China, the mutations in *EGFR* and *TP53* genes were analyzed on paraffin-embedded tumor samples related to lung cancer by the NGS method, and as a result of this study, the mutation rate in the *EGFR* gene was equal to 52% and gene *TP53* was reported as 28% (79). In a study conducted by Tsiatis et al. in 2010, *KRAS* gene mutations in paraffin-embedded lung adenocarcinoma tumor samples were investigated with three methods, including sequencing, and as a result of this study, about 63% of the samples had mutations in the *KRAS* gene (59). In a study conducted by Grosse et al. in Switzerland on lung cancer patients, mutation frequency in *KRAS* oncogene was investigated using sequencing and NGS methods. As a result of this study, the rate of mutation in the *KRAS* gene was reported as 34% (19). In a study conducted by Jang et al. in 2009 in Korea, the mutations of *EGFR* and *KRAS* genes were evaluated using the sequencing method in patients with lung adenocarcinoma. As a result of this study, about 10 and 25% of mutations were reported in *KRAS* and *EGFR* genes, respectively (80). In a study conducted by Tuononen et al. in Finland, the mutations of *EGFR* and *KRAS* genes in paraffin samples of lung cancer tumors in the number of 81 samples were investigated using the NGS method. As a result of this study, about 25% of patients had mutations in EGFR and 33% had mutations in *KRAS* gene (81). Other researchers have investigated the mutation frequency of the *KRAS* gene in lung cancer patients, including Tsao et al. in 2010 about 34% mutation, Kern et al. in 1994 about 36% mutation, Capelletti et al. in 2010, 27 Regarding the percentage of mutation, Zhao et al. reported 22% mutation, Schmid et al. 37% mutation, and finally Schiller et al. 24% mutation in the *KRAS* gene in lung cancer patients (58, 65, 67). In a study conducted by Carter et al. in 2015 in Taiwan, the V600E mutation of the *BRAF* gene was investigated in lung, colon and melanoma cancer samples using the NGS method. As a result of this study, the mutation rate of this gene in lung cancer was reported as 6% (82). There have been many studies on the mutation frequency in the *TP53* gene. In the study conducted by Zhao et al. in 2019, *TP53* gene mutations were investigated in 50 patients with non-small cell lung cancer using sequencing techniques. As a result of this study, about 45% of mutations in the *TP53* gene were reported, and most of these mutations were located in exons 5, 7, and 8 of the *TP53* gene (65). In the study conducted by Shajani et al. in 2018, *TP53* gene mutations in colon, lung and glioblastoma cancers were investigated using the NGS method. As a result of this study, about 36% of people with lung cancer had mutations in *TP53* (78). In a study conducted by Labbe et al. in 2017, *TP53* gene mutations were investigated in lung tumor samples using NGS and sequencing techniques. As a result of this study, about 40% of the mentioned samples had mutations in the *TP53* gene (83).

In this study, genomic susceptibility regions that are related to the incidence of lung cancer were discussed and investigated. Further clinical-laboratory study of these areas related to lung cancer in different populations can be a beginning for designing a lung cancer screening panel to identify susceptible and high-risk individuals. The high lethality of lung cancer as well as diagnosis in advanced stages has forced researchers to discover and investigate molecular markers for the early diagnosis of this disease and also to evaluate the response to treatment.. Meanwhile, among the gene-offending regions, the most altered genes were those pertaining to the *EGFR, KRAS, TP53*, and *BRAF* genes. Consequently, studying the mutations of these genes in lung cancer can be crucial for clinical prediction, screening, early disease diagnosis, and treatment response assessment. Stated differently, the detection of these mutations as clinical biomarkers in the form of a screening panel can be crucial for early diagnosis, treatment process facilitation (by assessing treatment resistance), mortality reduction, and a decrease in the material and spiritual damages associated with this illness.

Declarations

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Exploring Aspartic Acid D-repeat Polymorphism as a Potential Risk Factor for Primary Hip Osteoarthritis in the Iranian Population

Mohammad Qoreishy[1], Abdoreza Sajedi[2], Mostafa Qorbani[3], Mina Makvand[4], Roshanak Jazayeri[5*]

[1]Associated professor of Orthopaedic Surgery, Shahid Beheshti university of medical sciences, Tehran, Iran.
[2]Department of Orthopedics, Medical School, Shahid Beheshti University of Medical Sciences, Tehran.
[3]Non-communiable Diseases Research Center, Alborz University of Medical Sciences, Karaj, Iran.
[4]Department of Medical Genetics, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.
[5]Department of Genetics, Faculty of Medicine, Alborz University of Medical Sciences, Karaj, Iran.

Corresponding author: Roshanak Jazayeri, Department of Genetics, Faculty of Medicine, Alborz

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Abstract:
Background: The *ASPN* gene encodes a cartilage extracellular protein (Asporin) that is known to be involved in the pathological paths of osteoarthritis (OA). Many research efforts have explored the link between aspartic acid D-repeat polymorphism in the asporin (*ASPN*) gene and the risk of OA susceptibility, yet the findings are inconsistent. Our study involved a case-control analysis to examine the relationship between D allele polymorphism in asporin and primary hip osteoarthritis (HOA) among the Iranian population.
Methods: The asporin D repeat polymorphism was genotyped in primary HOA patients (N=70) and healthy controls (N=70). Each group consisted of 28 women and 42 men. Patients were classified into three subgroups based on the radiographic severity of osteoarthritis. Statistical analysis was performed on gender, severity, and primary HOA position.
Odds ratios (ORs) along with 95% confidence intervals (95% CIs) were utilized to assess the association between D-repeats in the ASPN gene and primary hip osteoarthritis.
Results: Three common D-repeat variants (D13, D14, and D15) of the *ASPN* gene were obtained. The most frequent allele in the patient group was observed at D13, while it was D15 among controls. In both cohorts, the least frequent allele was D14. Our findings indicate no statistically significant association between any D-repeats with primary HOA according to the sex of patients or the severity of the disease.
Conclusion: Our findings indicate that polymorphisms in the *ASPN* D-repeat are not linked to a higher risk of primary HOA in the Iranian population. However, future large studies are needed to validate these findings.

INTRODUCTION

HOA is an age-related form of OA and a leading cause of musculoskeletal disability. HOA is a degenerative joint disease that affects the articular surface of the joint. It is characterized as either primary (or idiopathic) or secondary in the elderly population (1). Incidence rate estimations by the World Health Organization (WHO) indicate that 10% of men and 18% of women aged over 60 years suffer from HOA (2). Furthermore, this disease's prevalence is expected to increase significantly shortly.

Symptoms related to HOA significantly affect patients' social and physical well-being and also represent a considerable economic burden on society. HOA is influenced by multiple risk factors such as body mass index (BMI), age, sex, and genetic background (3). The exact mechanism underlying the pathogenesis of OA remains unclear. However, numerous studies have investigated the link between aspartic acid (D) repeat polymorphism in the ASPN gene and the risk of developing knee osteoarthritis (KOA) and HOA across various

populations (4) The *ASPN* gene produces Asporin, a cartilage extracellular protein that belongs to the small leucine-rich proteoglycan (LRR) family.

The *ASPN* gene is cytogenetically located on human chromosome 9 within a specific gene cluster between 9q21.3 and 9q22 Asporin is a critical regulator of transforming growth factor beta 1 (*TGFb1*), and through an inhibitory mechanism, it affects *TGFb1*-induced gene expression, which negatively regulates chondrogenesis in cartilage (5). The N-terminal region of asporin consists of aspartic acid residues (D-repeat). The poly-Asp region is involved in binding calcium (3). Heterogeneity in the number of D-repeats in this region has been reported to be associated with susceptibility to osteoarthritis, although the results are inconsistent. The contradictions led us to investigate the validity of this association among Iranians. In addition, we will answer the question of whether this association with osteoarthritis is worth considering.

According to the principle of multifactorial disease, genetic background and environmental differences influence genetic diversity and phenotype between different ethnicities, which may lead to controversial results across studies in different populations. To our knowledge, no study has investigated the connection between *ASPN* gene Asp repeat polymorphism and primary Hip OA penetrance in the Iranian population. Therefore, we performed a case-control study based on a robust stratified analysis of gender and primary HOA severity rates with a reliable sample size to further elucidate the role of asporin D allele polymorphism in primary HOA susceptibility among the Iranian population. Multi-haplotype analysis (D13, D14, and D15 alleles) enabled us to increase the evidence for hip association.

MATERIALS AND METHODS

Patients

A case-control study was conducted with a sample size (N =70) for each group, of which 28 women and 42 men were regarded as control cases, as well as OA patients according to exclusion/inclusion criteria. The standard deviation for sample size calculation is obtained from the previous study of our team on knee osteoarthritis in Iran (6). All subjects in the study were of Iranian nationality and came from the same geographical area of the country (Tehran).

All procedures and clinical research were carried out in accordance with the ethical standards and subsequent amendments outlined in the declaration of Helsinki. All participants signed the written informed consent. Cases were defined as patients based on inclusion criteria including chronic pelvic pain, radiological verification, limitations in mobility, those without any history of inflammatory joint disease, inflammatory arthritis, joint dysplasia or congenital disorder, etc. Clinical symptoms and radiographic evidence of hip osteophytes and narrowing of the joint space were used to diagnose hip OA. All patients with hip OA were categorized into three grades according to the Tönnis classification. Tönnis grading scale of HOA (7, 8):

-Grade 1: This classification involves mild narrowing of the joint space, minor lipping at the joint margin, and slight sclerosis of either the femoral head or the acetabulum.

- Grade 2: This grade is characterized by the presence of small cysts in the femoral head or acetabulum, increased narrowing of the joint space, and moderate loss of femoral head sphericity.

-Grade 3: This classification includes large cysts, severe narrowing or complete obliteration of the joint space, severe deformity of the femoral head, and avascular necrosis (7). The controls were healthy people with normal radiographs of the hip joint space. (Table S1 gives information about the demographics of both groups). Samples were collected from Akhtar Hospital-Orthopaedic Surgery Center at Shahid Beheshti University of Medical Sciences. The Institutional Review Board Committee at Shahid Beheshti University of Medical Sciences in Tehran, Iran, approved the study.

DNA extraction

DNA was extracted from 10 ^{cc} of collected peripheral blood into an EDTA tube according to standard protocols (9). DNA degradation was assessed using 1% agarose gels. Protein contamination in all DNA samples was evaluated by the A260/A280 ratio, and reagent contamination was determined by the A260/A230 ratio using a NanoDrop ND 1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

Primer design and PCR

The polymorphic Asp repeat region was identified by sanger sequencing for exon 2 of the *ASPN* gene (described by Mustafa and colleagues in 2005) (10). Primers flanking the regions, including a minimum of 60 bp from the exon-intron boundary of exon 2 of the *ASPN* gene (NM_017680.5), were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The forward primer sequence is 5'- GCTTTGTGCTCTGCCAAACCC -3', and the reverse primer sequence is 5'-CACTGACATCCAAATGGACAC-3'.

These primers were synthesized by Macrogen in Korea. The amplification of target sequences was performed in an ABI thermal cycler (Applied Biosystem Veriti™ Thermal Cycler, Thermo Fisher, Waltham, MA, USA) using reaction volumes of 25 µL. Each reaction contained 50 ng of DNA, 1× PCR buffer, 2.5 mM MgSO4, 200 µM of each dNTP, 10

pmol of each forward and reverse primer, and 5 U of Taq DNA polymerase (Macrogen, Korea). The PCR cycle conditions were set as follows: an initial denaturation at 95°C for 5 minutes; 30 cycles of 30 seconds at 94°C, 1 minute at 60°C, and 30 seconds at 72°C; and a final extension at 72°C for 3 minutes.

Genotyping

All the PCR products were directly sequenced using the BigDye v3.1 Terminator Cycle Sequencing Kit and the 3130 Genetic Analyzer (Applied Biosystems, Foster City, California). Sequencing results were identified by CodonCode Aligner software (CodonCode Corp, Centerville, Massachusetts).

STATISTICAL ANALYSIS

All case-control qualitative data were presented by number and percentages. We performed a case-control study for the ASPN D13, D14, and D15 alleles and hip OA susceptibility to assess the strength of the association respectively by calculating odds ratios (ORs) and 95% confidence intervals (CIs). We used the Chi-square test or Fisher exact test to investigate the association between genotype and the presence of increased levels of hip osteoarthritis (Severity of radiopathological symptoms) (11). Statistical analyses were conducted using the SPSS version 17.0 statistical package (SPSS, Chicago, IL, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA). A p-value of less than 0.05 was deemed statistically significant, and all tests were two-sided. The variables, such as age and BMI, were compared between the case and control groups using the t-test and chi-square test.

RESULTS

Three different alleles (D13, D14, and D15) were identified in the study groups.

Table 1 displays the distribution of allele frequencies for each case-control study, categorized by sex both before and after stratification. According to the Tönnis grading scale of primary hip osteoarthritis, the patient group was classified into three groups: grade 1, grade

Table 1. Allele frequencies of the asporin D repeat in hip OA patients and controls from the Iran.

Alleles	D13	D14	D15	Total
All patients (n=70)	62 (44.3%)	20 (14.3%)	58 (41.4%)	140 (100%)
Female patients (n=28)	25 (17.8%)	8 (5.7%)	23 (16.4%)	56 (40%)
Male patients (n=42)	37 (26.4%)	12 (8.5%)	35 (25%)	84 (60%)
All controls (n=70)	53 (37.9%)	18 (12.9%)	69 (49.3%)	140 (100%)
Female controls (n=28)	22 (15.7%)	6 (4.2%)	28 (20%)	56 (40%)
Male controls (n=42)	31 (22.1%)	12 (8.5%)	41 (29.2%)	84 (60%)

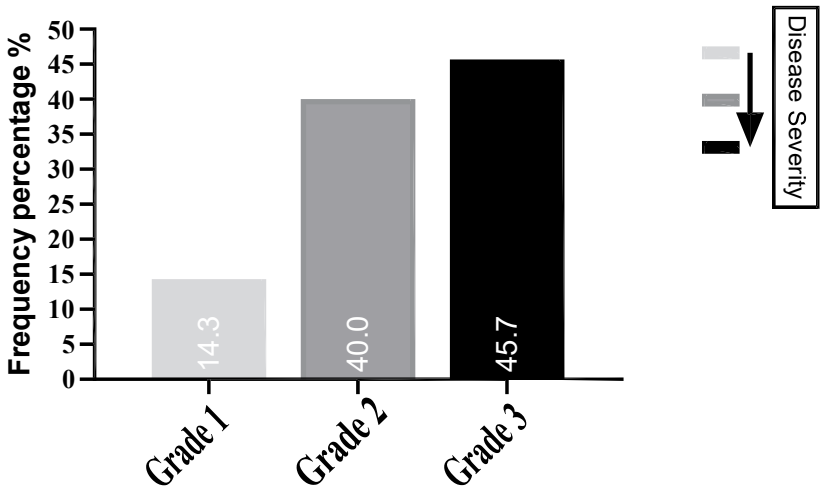

Fig 1. The bar plot demonstrates the value frequencies of the disease grades for hip OA group. The severity of the disease represented by color intensity. Most of the participants belongs to sever form of disease (45.7%)

Table 2. correlation between Asp allele repeats and Hip OA Tönnis grades in patients' group.

ALLELES	GRADE 1	GRADE 2	GRADE 3	PEARSON CHI-SQUARE	DF	OR
D13	40.00%	42.90%	25.00%	0.992	2	0.016
D14	20%	21.40%	21.90%	0.319	2	2.283
D15	40.00%	35.70%	53.10%	0.383	2	1.92

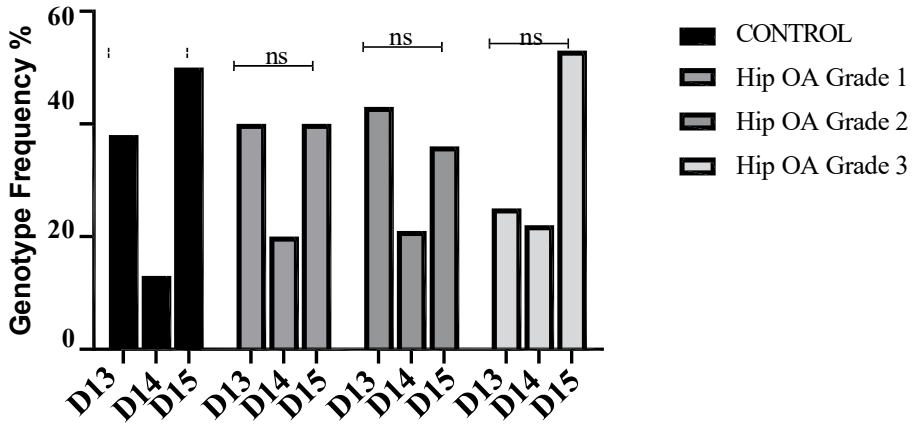

Fig 2. Bar plot represents association between Asp repeats genotype and Hip OA severity.no significant differences were observed between three alleles (D13, D14 and D15) and mild, moderate and severe form of Hip OA in patients group.

2, and grade 3. The frequencies of the disease grade classification for the patient group were 14.3% for grade 1, 40.0% for grade 2, and 45.7% for grade 3 (Fig 1).

The association between genotype and primary hip osteoarthritis severity was tested, and the following values were obtained p=0.319 for D13, p=0.992 for D14, and p=0.383 for D15 (Pearson Chi-Square test). The results demonstrated that there is no association between D allele repeats and primary Hip OA severity (Table 2 and Fig 2).

Targeted comparison analyses were performed for each allele frequency compared to all other frequencies combined, followed by a comparison of the most frequent alleles (D13, D15, and D14) in the patient group versus the control group before and after stratification according to sex and disease severity.

All alleles.

The overall allele frequency percentages in the study groups were (patient groups: D13:44.3%, D14: 14.3%, and D15: 41.4%) and (control group: D13: 37.9%, D14: 12.9%, and D15: 49.3%). Stratification was performed according to the sex criteria, and the results were; patient group: (D13:17.8% in women and 26.4% in men), (D14: 5.7% in women and 8.5% in men), and (D15: 16.4% in women, and 25% in men) and control group: (D13: 15.7% in women and 22.1% in men), (D14: 4.2% in women and 8.5% in men), and (D15: 20% in women and 29.2% in men). The findings showed that there were no significant differences ($P \geq 0.05$) in allele frequencies between primary HOA patients and controls, both with and without stratification.

D13 vs Other Alleles Combined

No significant differences were observed between the two groups either before or after stratification. (P = 0.27; OR=1.3; 95% CI, 0.81-2.1).

D14 vs Other Alleles Combined

There were no significant differences noted between the two groups, both before and after stratification. (P =0.72; OR=1.13; 95% CI, 0.57-2.24).

D15 vs Other Alleles Combined

Significant differences were not identified between the two groups, both before and after stratification. (P = 0.18; OR=0.72; 95% CI, 0.45-1.16).

D13 vs D14

No significant differences were found, regardless of whether stratification was applied.

D13 vs D15

No significant differences were found, regardless of whether stratification was applied.

D14 vs D15

No significant differences were found, regardless of whether stratification was applied.

Overall, there were no significant differences between the two groups, neither before and after the stratification nor in the status of radiological severity in

Table 3. Association of the D-repeat asporin polymorphism with Hip OA in the Iranian population

GROUPS COMPARED	D14 VS. D15			D15 VS. D13			D15 VS. OTHERS		
	95%CI	*P* value	OR	95%CI	*P* value	OR	95%CI	*P* value	OR
All PATIENTS(N=70) *VERSUS* ALL CONTROLS(70)	0.63-2.73	0.45	1.32	0.43-1.19	0.2	0.72	0.45-1.16	0.18	0.72
FEMALE PATIENTS(N=28) *VERSUS* FEMALE CONTROLS(N=28)	0.26-4.91	0.85	1.14	0.22-1.42	0.22	0.057	0.26-1.46	0.27	0.62
MALE PATIENTS(N=42) *VERSUS* MALE CONTROLS(N=42)	0.76-4.63	0.17	1.87	0.42-1.78	0.7	0.87	0.39-1.43	0.38	0.75
GROUPS COMPARED	D14 vs. D13			D13 vs. others			D14 vs. others		
	95%CI	*P* value	OR	95%CI	*P* value	OR	95%CI	*P* value	OR
ALL PATIENTS(N=70) *VERSUS* ALL CONTROLS(N=70)	0.45-1.98	0.89	0.95	0.81-2.1	0.27	1.3	0.57-2.24	0.72	1.13
FEMALE PATIENTS(N=28) *VERSUS* FEMALE CONTROLS(N=28)	0.14-2.90	0.57	0.65	0.7-4.15	0.23	1.71	0.21-3.56	0.85	0.87
MALE PATIENTS(N=42) *VERSUS* MALE CONTROLS(N=42)	0.65-4.11	0.29	1.63	0.49-1.86	0.91	0.96	0.76-4.07	0.18	1.76

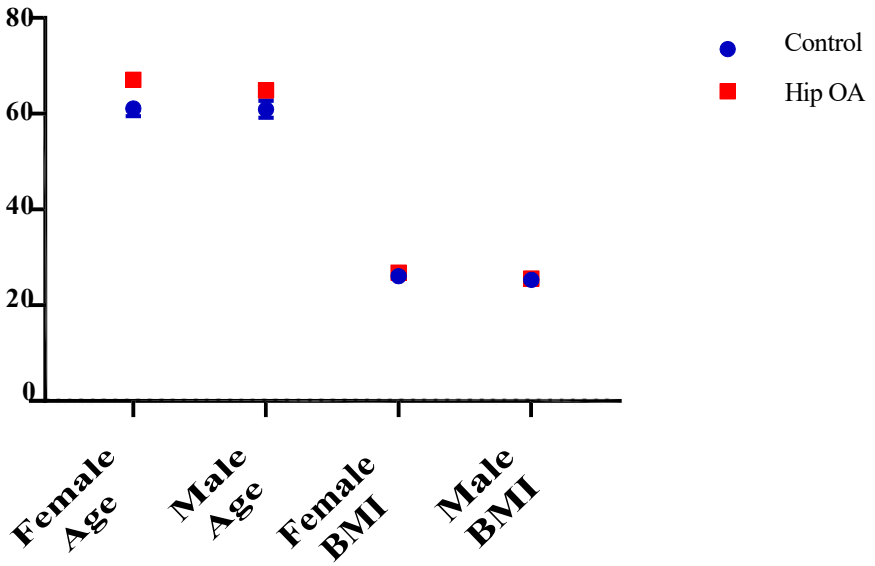

Fig 3. Dot plot demonstrates independent samples t-Test results for age and BMI by mean and variances between sexes in two cohorts. both populations (case and control) for each group located in close area and no significant differences were observed.

the patient group. (Table 3 demonstrates the statistical values). Using a *t*-test demonstrated that there was no difference in the outcome variables (age and BMI) between the primary hip OA patients and healthy control groups (Fig 3).

DISCUSSION

The present study demonstrates that there is no association between D13, D14, and D15 alleles and increased primary Hip OA risk in the Iranian population.

In recent years, several studies have paid particular attention to the correlation between asporin D- repeat polymorphisms and OA susceptibility in different populations. D-repeat is the unique aspartate-rich N terminus region that expands in exon 2 of the *ASPN* gene and encodes a cartilage extracellular protein.

Previously, it has been reported that *ASPN* gene polymorphism has an impact on OA disease and that asporin expression levels differ between various populations such as Caucasians vs. Japanese (12).

The D14 allele is the least common in the entire cohort (14.3% for patients and 12.9% for controls) However, the control and patient groups differ in that D13 is the most prevalent allele in patients, followed by D15, whereas D15 is the most frequent, followed by D13, in controls. The data regarding D14 allele frequency are consistent with our previous report on KOA among the Iranian population (6).

Reports vary regarding the most prevalent allele; D13 has been previously identified as more common among controls, while D14 is often found more frequently in patients within the UK Caucasian population (10). This demonstrates the different genetic diversity between Iranians and Europeans. However, meta-analyses by Sobhan et al. in eleven case-control studies in ten publications implied that D13 is more prevalent in case and control groups, whereas D14 is less common in both groups(3), similar to our results about the D14 allele.

Previously, D13 was reported to be associated with low OA risk and, thus, was considered a protective allele of Greek origin (13). This finding, however, was duplicated in a Japanese study (14). Also, gender-wise stratification indicated a high risk for HOA among women, and it was believed that D13 was a protective marker in men in the Romanian population (15). A meta-analysis by Wang. H et al. performed using twelve qualified studies from various ethnicities confirmed the critical role of the D13 allele as a protective marker in OA (4). Subsequently, the under-representation of D13 in OA patients was observed in the Han Chinese population (16). Hence, several studies from different populations have consented that the D13 allele is associated with a decreased risk for OA. However, our study detected no significant difference in allele frequencies between the primary HOA cohort and controls. Other meta-analyses in Asians and Europeans (3, 17) are consistent with our results. Moreover, functional studies demonstrated no significant difference in the inhibitory effects on TGF-beta between D13 and other alleles (16), which strongly supports our findings. Taken together, we assumed that the D13 allele does not have a major impact on primary HOA susceptibility in the Iranian population.

Regarding the D14 allele, the high frequency has been observed in developmental dysplasia of the hip (DDH) in the Han Chinese population and is claimed to be associated with early-onset OA (16). Furthermore, the over-representation of the D14 allele was reported as being associated with HOA and increased alongside disease severity, which is an outcome of the greater inhibition of TGF-beta for D14 (18). A high expression of the D14 allele was reported in OA cartilage tissue, which could mediate the suppression of the TGF-beta signaling pathway (13). Also, a meta-analysis revealed that D14 is a prominent risk factor for KOH in Caucasians (17). This result was duplicated in Caucasian and Japanese populations (19). Similarly, the D14 allele was associated with the risk of KOA development in Japanese and Chinese populations. In support of the above-mentioned reports, D14 is a common risk allele for KOA among Asians and male Caucasians (10, 20). In contrast with these data, our research team previously implied a protective role of the D14 allele among female KOA patients in the Iranian population (6). Moreover, D14 was associated with a high risk for HOA in men but was a protective marker in women. In a previous study, D14 had a significant association with increased radiological severity in both sexes in the Romanian population. (15) These data are inconsistent with our results, as we detected no significant association between D14 and primary HOA in the Iranian population. Previous meta-analyses of Europeans and Asians support our findings (3, 17). Considering functional studies and controversial results in various populations, we assume these findings could imply the ethnic dependence of the D14 allele's role in OA susceptibility.

The D15 allele was reported either to have an association with OA risk via a different mechanism, as a risk factor or to have a protective role in the Mexican and Iranian populations (3, 5). Our research team demonstrated that the D15 allele is a risk factor for KOA in Iranian female patients (6). However, this association is not confirmed for primary HOA in the same population in the present study.

Ultimately, our findings are different from previous investigations. Nevertheless, some studies support our results by indicating a negative association between OA susceptibility and D-repeat alleles

in different populations (21-23). Meta-analyses performed by Sobhan, Nakamura, Wang, H et al. and AA Brisola, et al. similarly reported no association for D-repeat in asporin and OA among Europeans and Asians (3, 17, 24, 25). Additionally, meta-analyses by Xing et al. suggest that the *ASPN* D-repeat polymorphism may not be a significant marker for KOA susceptibility in both Caucasian and Asian populations (21). As mentioned above, there are some discrepancies between reports about the link between the D-repeat allele and OA. Our results do not support the connection between susceptibility to primary HOA and D allele polymorphism. Generally, the KOA is more prevalent than the HOA (26), and so most research is focused on KOA. Thus, the problem of making valid comparisons across available documents is one of the pivotal limitations of studying HOA. Moreover, OA is a multifactorial disorder that is influenced by genetic background, age, sex, and environmental factors. These facts could explain the discrepancies between the results of research work conducted in different populations. The advantages of the present study, which due to the high level of consanguinity in Iran, could be more prominent in the genetic role compared to other nongenetic factors in this study.

Our study addresses a significant gap by investigating the *ASPN* gene's D-repeat polymorphism in the Iranian population, previously underrepresented in osteoarthritis genetics research. Despite the smaller sample size, our findings contribute preliminary insights into the unique genetic landscape of this group, highlighting the importance of including diverse populations in genetic research. This approach not only helps validate or challenge existing data but also enhances understanding of osteoarthritis's multifactorial nature, paving the way for future research and potential precision medicine advancements.

CONCLUSION

The findings suggest a lack of an association between aspirin D allele polymorphism and primary HOA susceptibility among the Iranian population. This is the first assessment of the association between primary HOA and D polymorphism in the *ASPN* gene in Iran. However, further investigations of larger populations and involving diverse ethnic cohorts are required to confirm the role of asporin genetic variation on primary HOA risk.

Abbreviations

ASPN = asporin, CI = confidence interval, LRR = leucine-rich, KOA = knee osteoarthritis, primary HOA = primary Hip osteoarthritis, OA =osteoarthritis, OR = odds ratio, TGF-b =transforming growth factor-b, D = aspartic acid, DDH = developmental dysplasia of the hip.

Statements and Declarations

Consent to participate and Publication

All participants read and signed the consent form.

Availability of data and materials

The data supporting the findings of this study can be obtained upon request from the corresponding author, R Jazayeri. These data are not publicly accessible due to restrictions such as containing information that could compromise research participant privacy.

Ethics approval and consent to participate

Written informed consent has been obtained from all participants in the study.

Conflicts of Interest: M Qoreishy, A Sajedi, M Qoreishi, M Makvand, and R Jazayeri declare that they have no conflict of interest.

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Authors' contributions

M Qoreishi, A Sajedi, M Qorbani, M Makvand, and R Jazayeri were instrumental in designing and implementing the research, analyzing the results, and contributing to the writing of the manuscript.

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Introduction of Spinal Muscular Atrophy Disease and the Latest Treatment Approaches based on Gene Therapy

Raziyeh Gorji[1*], Shinoo Minaei[2], Saeed Homaei[2], Mitra Rashidi[3]

[1]Department of Molecular Genetics, Faculty of Basic Sciences and Advanced Technologies in Biology, University of Science and Culture, ACECR, Tehran, Iran.
[2]Department of Biology, Faculty of Biological Sciences, East-Tehran Branch, Islamic Azad University, Tehran, Iran.
[3]Department of Microbiology, Faculty of Biological Sciences, East-Tehran Branch, Islamic Azad University, Tehran, Iran.

**Corresponding author: Raziyeh Gorji, Department of Molecular Genetics, Faculty of Basic Sciences and Advanced Technologies in Biology, University of Science and Culture, ACECR, Tehran, Iran. Email: razigorji1234@gmail.com.*

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Abstract:

Spinal muscular atrophy (SMA) is a prevalent autosomal recessive disorder characterized by gradual weakening of the skeletal and respiratory muscles, resulting in substantial impairment. The illness is a result of genetic abnormalities in the survival motor neuron 1 (*SMN1*) gene, which leads to a reduction in the SMN protein and subsequently causes the degeneration of lower motor neurons. Gene therapy is a method that has the potential to cure or prevent uncommon monogenic illnesses by substituting a defective gene with a functional one. Gene therapy is particularly suitable for monogenic illnesses since it has the ability to correct abnormalities in a single gene. Currently, Nusinersen, risdiplam, and onasemnogene abeparvovec are the only officially sanctioned treatments for SMA that have the ability to influence the course of the illness. The purpose of this analysis is to examine and analyze their mechanisms of action, impacts, and potential safety issues. Nusinersen and risdiplam function by altering the *SMN2* gene product, whereas onasemnogene abeparvovec operates by introducing copies of the *SMN1* gene into cells. In this article, we briefly describe the pathogenesis and treatment strate, gies of SMA.

INTRODUCTION

Spinal muscular atrophy (SMA) is a genetic disorder characterized by the degeneration of neurons in the spinal cord, resulting from a lack of survival of motoneuron protein (SMN). The reduction in the synthesis of the whole SMN protein occurs due to an insertion or deletion of the *SMN1* gene situated on the 5q13.2 chromosome. The SMN protein is crucial in the development of SMA (1). It has a role in preserving cellular homeostasis. SMN is accountable for the accurate assembly of the spliceosome and the production of ribonucleoproteins. It has the potential to facilitate the formation of RNA complexes with the diverse proteins necessary for effective transportation or localized protein synthesis. SMN regulates the production of several components that participate in DNA repair or possess anti-apoptotic properties. Deficiency of the SMN protein results in the deterioration of -motoneurons located in the anterior horns of the spinal cord, which in turn leads to progressive muscle wasting (2).

Furthermore, in more severe manifestations of SMA, there is evidence of involvement of other cell and tissue types, leading to the emergence of symptoms that are not directly connected to motor neurons. There is a notable decrease in the quantity of chondroblasts in the hypertrophic zone of the growth plate, which leads to hindered bone growth. Angiogenesis and vascular maturation abnormalities caused by SMN deficiency exacerbate motor neuron hypoxia, therefore playing a role in the development of SMA. SMA is a degenerative and diverse condition. Irrespective of the specific kind, SMA has a substantial impact on patients and impacts them in intricate ways. In the absence of proper treatment, it results in muscular debility, paralysis, and ultimately, in extreme instances, fatality due to respiratory insufficiency (2).

The SMA phenotype is classified into four classes of severity (SMA I, SMA II, SMA III, SMA IV) according to the age at which symptoms appear and the level

of motor function attained. There are four types of SMA. Type 1 is the most severe, characterized by the patient's inability to sit. Type 2 involves the patient being unable to walk without assistance. Type 3 allows for some movement capabilities. Type 4 refers to adult-onset SMA (3).

The clinical significance of different kinds of SMA is mostly determined by the quantity of *SMN2* copies, while other genetic or environmental factors have a minimal impact. As the number of *SMN2* copy mutations increases, the likelihood of having a severe phenotype also increases. Therefore, when the number of *SMN2* types increases, the clinical severity also increases. Untreated, this condition will lead to significant impairments in motor function, like the inability to walk; a heightened likelihood of respiratory problems requiring some level of ventilatory assistance; a greater susceptibility to orthopedic issues such as painful contractures and scoliosis; and a decreased lifespan. SMA Type 1 accounts for 45% to 60% of all cases of SMA, making it the predominant form of the disease. Individuals diagnosed with SMA Type 1 who possess two copies of the *SMN2* gene have a very unfavorable outlook. Typically, these patients exhibit symptoms of SMA before reaching six months of age, which is characterized by their inability to sit. Regrettably, these infants generally do not live beyond the age of two without substantial reliance on mechanical ventilation and nutritional assistance. The *SMN2* gene is an extremely similar duplicate of the *SMN1* gene and is therefore regarded as a phenotypic modulator of the SMA disease. A higher quantity of *SMN2* gene copies corresponds to a less severe manifestation of SMA in clinical presentation (3).

Pathogenesis of SMA

The gene responsible for causing SMA is located in the q13 area of chromosome 5. SMA is a condition that occurs due to a mutation or deletion in the survival of *SMN1*gene, leading to the inability to produce the survival motor neuron (SMN) protein. SMN is a ubiquitously expressed protein that is involved in the formation of the U-rich small nuclear ribonucleoprotein (U snRNP) and aids in the transportation of axonal mRNAs (4). A recent study has provided a detailed summary of the activities of the SMN protein. The 5q13 region is characterized by a virtually symmetrical structure resulting from the duplication of a long stretch by inversion. *SMN1* is situated on one side of the telomere within this area. On the centriole side of this area, there is a parallel counterpart of *SMN1* called survival of motor neuron gene 2 (*SMN2*) (5). *SMN2* only produces a small quantity of the functional SMN protein, which is insufficient to make up for the decrease in SMN induced by the deletion of *SMN1*. *SMN1* and *SMN2* exhibit a high degree of similarity, with just 16 known variations in their base sequences. The main distinction is in the sixth nucleotide of exon 7; SMN1 has a cytosine (C), while *SMN2* possesses a thymine (T) (6). While this variation in a single DNA nucleotide does not impact the encoding of amino acids, it does influence the process of exon 7 splicing. Consequently, the majority of the gene products produced by the *SMN1* gene consist of complete messenger RNA molecules that include exon 7. In contrast, the gene products of *SMN2* are messenger RNA molecules that lack exon 7 (Δ7). The Δ7 mRNA experiences a displacement of its stop codon, leading to the production of a shortened SMN protein that lacks functionality and is very unstable. Thus, enhancing the incorporation of exon 7 of *SMN2* is a viable approach for treating SMA (6).

The degree of SMA is directly influenced by the quantity of SMN protein present. In general, individuals with moderate SMA possess a greater number of *SMN2* gene copies compared to those with severe SMA. This is because a larger copy number of *SMN2* results in elevated quantities of SMN protein. Furthermore, many regulations, including point mutations, might impact the severity of SMA patients by modifying the incorporation of exon 7. The precursor mRNA of *SMN2* contains several cis-acting splicing regulatory elements, including exonic splice silencer (ESS), exonic splicing enhancer (ESE), intronic splicing silencer (ISS), and intronic splicing enhancer (ISE). These components bind to trans-acting factors and regulate the inclusion of exon 7 (7).

Options for the treatment of SMA

Throughout history, there have been several therapy methods available for SMA; however, only a limited number of them are applicable to patient treatment. However, they are practical and can be transformed into innovative medications in the future. Our approach to developing medications against SMA follows the sequence of "Gene-RNA-Protein-Cell-Tissue." Numerous exemplary evaluations have provided detailed descriptions of several of these tactics.

Gene-Targeted Therapy: This treatment involves introducing intact SMN-encoding genes into cells to induce their expression, resulting in a substantial synthesis of SMN protein. This procedure effectively transferred the *SMN1* gene to motor neurons in several animal models by using self-complementary adeno-associated virus 9 (scAAV9), resulting in a significant extension of the lifespan in mice models of SMA. The medicine abeparvovec-xioi, developed by Novartis' AveXis, gained approval in the US in 2019 and was introduced to the EU in 2020 using this approach. Another approach is to enhance the transcriptional expression of *SMN2*, for instance by using the histone deacetylase inhibitor SAHA. This method has successfully resulted in elevated levels of SMN protein in animals. Nevertheless, this method has not achieved success in clinical studies so far (8, 9).

Therapy aimed at modifying splicing: This treatment approach seeks to enhance the production of the functional SMN protein by increasing the incorporation of *SMN2* exon 7. There are now two medications that have been authorized for sale. The first drug is called nusinersen, while the second drug, which was introduced more recently, is called risdiplam (10).

Protein stabilisation therapy: Cellular proteins maintain

a state of dynamic equilibrium, where their synthesis and degradation are balanced. This approach maintains the protein abundance of SMN by either inhibiting protein breakdown or facilitating the continuation of protein synthesis in spite of the existence of stop codons. The main drugs utilized in this therapeutic strategy are indoprofen and aminoglycosides. Specific small-molecule medicines have similar effects and possess the capacity to greatly augment the production of the SMN protein in cells. Nevertheless, their influence on the lifespan of SMA mice is only slightly enhanced (11, 12).

Substitution of cells: This method seeks to replace healthy neurons and restore the nerve supply to muscles. An alternative is to prompt the differentiation of embryonic stem cells or induced pluripotent stem cells (iPSCs) into neurons or neural stem cells in a controlled laboratory environment, with the intention of later transplanting these cells into the body. An alternative approach involves reprogramming fibroblasts from patients with SMA into iPSCs, and then genetically correcting them in a laboratory setting. These iPSCs may then be further induced to differentiate into motor neurons, which can be transplanted back into the same patient. This strategy is suitable for nearly all neurological diseases; however, present innovation has not sufficiently progressed for the management of SMA (13, 14).

Neuroprotective therapy: This technique is designed to provide nutrients and safeguard neurons. Riluzole is the primary medicine used in this treatment. Riluzole can enhance the survival of neurons and the development of axons by reducing the harmful effects of glutamate and increasing the production of neurotrophic factors. It has been approved for the management of amyotrophic lateral sclerosis, which is another type of neurodegenerative disease (15). Nevertheless, riluzole had little impact on the average lifespan of SMA animals and did not provide any defense against the loss of proximal axons. Despite the positive outcome of a clinical study with a limited number of participants, demonstrating potential benefits of riluzole for children with SMA, there is a lack of published findings from subsequent trials. In summary, this treatment approach lacks specificity and is unable to enhance SMN protein levels near their origin, thereby limiting its effectiveness in treating the condition (15).

FDA-approved treatments for SMA

Nusinersen

Nusinersen was authorized by the Food and Drug Administration (FDA) and the European Medical Agency (EMA) in December 2016 and June 2017, respectively, as one of the first medications for the treatment of SMA. This medication is classified as an antisense oligonucleotide (ASO) and works by suppressing the splicing process in the intron 7 of the *SMN2* gene, resulting in the incorporation of exon 7 into the *SMN2* mRNA transcripts (16). The ISS-N1 sequence is situated in exon 7, after the 5'ss. This segment hinders the inclusion of exon 7 by attaching to positions 10-24 of intron 7. Evidence demonstrates that the use of nusinersen may enhance the concentration of the SMN protein by inhibiting ISS-N1 and, as a consequence, inhibiting hnRNP. This leads to the inclusion of exon 7 in *SMN2* transcription. The progressive nature of the illness is decelerated by the augmented presence of functioning SMN protein. The medication is given intrathecally, meaning it is injected into the spinal canal. During the initial loading phase, it is supplied four times over two-month. In the maintenance period, it is given every four months. The dosages are specifically designed to affect the central nervous system. It is important to note that if the medication is given subcutaneously (under the skin) or intravenously (via a vein), it does not pass across the blood-brain barrier. An extensive study was required to establish the efficacy of nusinersen (17).

The safety of nusinersen has been assessed in several trials involving persons with SMA. Based on a thorough analysis of the data, no particular safety issues were found that could be directly linked to the medicine. The primary adverse effects associated with nusinersen are upper and lower respiratory tract infections, atelectasis, constipation, headache, back discomfort, and post-lumbar puncture syndrome. Two issues have been noted while using other experimental antisense oligonucleotides, and the FDA has cautioned about the potential occurrence of thrombocytopenia and renal toxicity with nusinersen. If there is a clinical need, it is suggested to do platelet count, prothrombin time, and spot urine protein tests before administering the treatment. This drug does not have any documented significant interactions. Due to the very short length of several clinical trials (less than 18 months), continuing investigations are being conducted to assess the long-term implications of drug interactions and safety hazards associated with the treatment. Nevertheless, nusinersen must be administered at intervals of 4 months, with an initial cost of $750,000 for the first year, followed by an annual cost of $350,000 thereafter (18, 19).

Clinical trials with nusinersen for SMA

In 2016, the first human clinical trials of nusinersen in children with SMA types 2 and 3 and type 1 (CS3a study were reported. The promising outcomes provided the basis for three Phase 3 trials: ENDEAR, CHERISH, and NURTURE. The approval of nusinersen as the first disease-modifying medication for SMA was mostly based on the outcomes of the first two trials (20, 21).

The ENDEAR (CS3B) trial was a multicenter worldwide research that used a randomized, double-blind, sham-controlled design. The study focused on babies diagnosed with SMA type 1 who had two copies of the *SMN2* gene and were under 7 months of age at the time of enrollment. A total of 121 babies with symptoms participated in the trial, including 80 in the nusinersen group and 41 in the control group (which did not receive any medication) (22). The trial was halted prematurely due to a prespecified interim analysis that revealed a much greater proportion of motor-milestone responders in the treatment group

compared to the control group, with percentages of 41% and 0% respectively. Ultimately, the treatment group exhibited a percentage of 51%, whilst the control group maintained a percentage of 0%. Multiple participants achieved important motor milestones with therapeutic implications, including acquiring head control, the ability to turn over, and attaining unsupported sit. During the research period, those who received therapy had significantly better overall survival and also had more time before needing permanent assisted breathing. Infants with a shorter duration of the illness had more favorable results compared to those with longer periods of illness, suggesting that initiating therapy soon is essential in SMA type 1 (22).

The CHERISH (CS4) study was a multinational, double-blind, sham-controlled clinical trial conducted in children between the ages of 2 and 12 who have SMA type 2. The trial comprised a total of 126 children who exhibited symptoms, with 84 children randomized to the nusinersen group and 42 children assigned to the control group. In addition, they were classified according to their age at the time of screening, either as under 6 years old or 6 years old and above (23). Just 16% of the population were above the age of 6. Every person exhibited the capacity to sit autonomously and attained notably high initial ratings on both the HFMSE and RULM evaluations. The experiment was terminated early due to ethical considerations, namely because of an interim analysis. The change in HFMSE score from the initial measurement was 5.9 points, as calculated using the least- squares mean approach. In the end, there was a difference of 4.9 points between the two groups. The nusinersen group had an average rise of 3.9 points, while the control group had an average drop of 1.0 points. A clinical significance is indicated by suggesting a minimum improvement of 3 points on the HFMSE scale (23).

In December 2016, the US FDA approved nusinersen as the initial drug for treating SMA, making it the first of its kind (24). In August 2021, nusinersen is accessible in 22 European countries. It is available without any limitations in 14 countries, while in 7 countries it is only accessible to particular forms of spinal muscular atrophy (SMA) and/or with some limits. Additionally, in 1 country it may be accessed via an early access program. Nusinersen has obtained regulatory authorization for its utilization in all types of SMA, encompassing all age cohorts and illness phases. As of March 2021, Biogen reported that over 11,000 patients worldwide have undergone nusinersen therapy (24).

The research observed individuals diagnosed with spinal muscular atrophy (SMA) types 2 and 3, ranging in age from 2 to 15 years at the beginning of the trial. These individuals were first included in the CS2 Phase 1/2 study and then continued their participation in the CS12 Phase 2 open-label extension study. The findings demonstrated sustained enhancements in motor abilities and steady disease activity over a span of roughly 3 years (25). Similarly, the CS3A study concludes that a significant fraction of the SMA type 1 therapy group saw a long-lasting clinical improvement, which is similar to the findings of the ENDEAR research, for a median follow-up period of 36.2 months. Upon completion of the trial, 75% of the subjects were still living. EMBRACE was a Phase 2 research that included 21 symptomatic babies and children who were not eligible for the ENDEAR or CHERISH investigations. The duration of the blinded 14-month portion was reduced according to the interim findings of the ENDEAR study. Phase 2 of the study had a total of 20 participants who were enrolled in an open-label research trial that spanned a duration of 28 months. All participants, with the exception of one, who were administered nusinersen in all phases of the study, met the HINE-2 motor-milestone response criteria, irrespective of their age at the onset of SMA (26).

Zolgensma

Zolgensma (AVXS-101, Onasemnogene Abeparvovec) is the second medicine that has been authorized for the treatment of SMA. The FDA granted approval to Zolgensma in 2019 for the therapeutic intervention of genetic testing-diagnosed SMA in children below the age of 2. Gene replacement therapy (GRT) was developed as a result of understanding the genetic cause of the condition (27). Onasemnogene abeparvovec is a gene therapy that employs an adeno-associated virus as the vector to transport a fully functioning copy of the human *SMN* gene to targeted motor neuron cells. Administering the SMN protein intravenously once leads to its expression in the motor neurons of a kid. This expression improves muscular mobility, function, and the child's overall survival in the case of SMA. The ongoing and completed clinical trial of onasemnogene abeparvovec has shown that it is safe and effective. The experiment had 36 pediatric infants diagnosed with infantile-onset SMA, ranging in age from around 2 weeks to 8 months at the beginning of the study. It is important to mention that, unlike nusinersen, Zolgensma can pass across the blood-brain barrier. Additionally, a single injection of Zolgensma during a 1-hour intravenous infusion is enough to achieve widespread expression of the SMN protein throughout the body. In contrast to the typical disease progression seen in kids with infantile-onset SMA, those who received treatment with onasemnogene abeparvovec showed a notable enhancement in their capacity to achieve developmental motor milestones, including improved head control and the ability to sit unassisted. Onasemnogene abeparvovec often causes increased levels of liver enzymes and vomiting as adverse effects. Therefore, it is necessary to closely monitor the liver function of patients for a minimum of 3 months after the administration of onasemnogene abeparvovec (28-30).

Currently, more than 12 unique serotypes of adeno-associated viruses (AAVs) have been discovered. Their cell tropism varies, and this variance is governed by the kind of viral surface proteins they possess. Furthermore, they vary in terms of their transduction efficiency and capacity to elicit an immunological response. AAVs have the capacity to infect both

actively dividing and non-dividing cells, while also showing low immunogenicity and decreased toxicity. The aforementioned traits render AAVs well-suited for the sustained expression of transgenes in clinical environments. Furthermore, a considerable proportion of AAVs have shown the capacity to efficiently deliver genetic material into both neurons and glial cells. This capacity has facilitated the creation of vectors obtained from AAVs for the treatment of neurodegenerative diseases (29).

Clinical trials with Zolgensma

The START clinical study was done in the United States to evaluate the effects of a single dose of onasemnogene abeparvovec on newborns diagnosed with SMA type 1. The research included a sample of 15 newborns, with a mean age of 6.3 months, all of whom have both copies of *SMN2*. Out of these newborns, 12 were given a high dosage of onasemnogene abeparvovec, whereas 3 got a low dosage. The experiment was conducted as a Phase 1 trial and the findings were compared to those of a historical cohort. Following a gene transfer period of 20 months, 11 out of the 12 toddlers who were administered a substantial amount of onasemnogene abeparvovec were capable of sitting independently and feeding themselves autonomously. The treatment resulted in the individual's survival and achievement of motor milestones and motor abilities that deviates from the expected development of the disease (28).

An open-label Phase 3 research, known as CL-303 (STR1VE-US), was conducted in the United States. The research included 22 infants aged below 6 months who were diagnosed with SMA type 1 and had 2 copies of the *SMN2* gene. The research was a single-arm trial conducted over a period of 18 months, including the injection of a single dosage of Zolgensma by intravenous route. At 14 months old, 20 out of 22 patients were alive and did not need permanent ventilation, while 18 out of 22 patients were fully free from the need for ventilatory assistance. By the age of 18 months, 59% of the individuals included in the research demonstrated the ability to sit alone for a minimum of thirty seconds. In comparison, none of the participants in the control group achieved this milestone. Additionally, 68% of the participants did not need assistance with feeding. After one month of treatment, the participants' CHOP INTEND scores had increased, with an average improvement of 6.9 points from the initial score (31).

The STR1VE-EU (CL-302) trial was conducted in Europe to investigate persons diagnosed with spinal muscular atrophy type 1 and possessing either 1 or 2 copies of the *SMN2* gene. The study consisted of 32 patients who had a more serious pattern at the start of the trial, in contrast to the START and STR1VE-US investigations. The patients' survival rate was similar to that of the STRIVE-US research, with 97% (31/32) of kids surviving without the need for permanent ventilatory support at 14 months of age, and 39% of patients not needing any daily ventilatory assistance. After the research, 44% of participants were able to sit independently for at least 10 seconds. The observed impact is less pronounced compared to the STR1VE-US trial, perhaps due to starting disparities in the study populations. Nevertheless, there was a comparable rise in CHOP INTEND scores, exhibiting an average shift of 6.0 points from the first measurement (32).

Risks of Zolgensma

Safety concerns include potential complications such as impaired liver function, low platelet count, clotting disorders affecting small blood vessels, and increased levels of troponin-I. Thrombotic microangiopathy is an uncommon, sudden, and potentially fatal illness, marked by low platelet count and the destruction of red blood cells in small blood vessels, with low platelet count being a prominent characteristic. Due to the potential for liver damage and other immune-related negative effects after AAV-based gene therapy, it is advised to provide preventative systemic corticosteroids before and after giving onasemnogene abeparvovec (33, 34). The intravenous injection of onasemnogene abeparvovec has the potential to influence several kinds of cells. Side effects have been recorded in multiple cell types and tissues, such as thrombocytes in the blood, as well as the liver, kidneys, and heart. Typical adverse effects include of emesis and increased levels of liver enzymes. While typically temporary and not medically significant, it is crucial to acknowledge the possibility of liver failure due to hepatotoxicity resulting from a hyperinflammatory response. It is recommended to monitor liver enzymes for a minimum of 3 months after receiving the onasemnogene abeparvovec infusion. No central nervous system (CNS) side effects have been seen in people. However, toxicity in the dorsal root ganglion has been seen in nonhuman primates when administered intrathecally. Hence, it is important to closely observe this as a potential unfavorable occurrence. Inpatient therapy may be required for these immune-mediated side effects after medication, along with the administration of intravenous steroids and other immunosuppressants (35).

Small-molecule compounds

Risdiplam is a small compound that, similar to nusinersen, alters the process of *SMN2* pre-mRNA splicing. This molecule easily passes across the blood-brain barrier and is taken orally once a day, allowing it to be distributed and absorbed effectively in both central and peripheral tissues. Risdiplam, marketed as Evrysdi®, is the first orally administered medication specifically designed for the treatment of SMA. It has received approval in several countries throughout the globe. It is authorized for treating spinal muscular atrophy (SMA) in children who are at least 2 months old in the United States and the European Union. In the European Union, this authorization is specifically for treating a kind of SMA called 5q-autosomal recessive SMA, which includes SMA types 1, 2, or 3, or SMA with one to four copies of the survival motor neuron 2 (*SMN2*) gene. Risdiplam functions as a regulator of *SMN2* pre-mRNA splicing, resulting in an augmentation of the production of intact SMN protein. The absence of this protein is responsible for

the development of SMA's pathological processes. Risdiplam shown a substantial improvement in motor function for newborns diagnosed with SMA type 1 and for people aged 2-25 years with SMA type 2 or 3 throughout phase 2/3 clinical studies. The motor enhancements were sustained for duration of up to 2 years by the administration of risdiplam. Risdiplam had overall good tolerability, demonstrating a favorable balance between benefits and risks. Risdiplam, being an oral medication, offers a simple and beneficial therapy choice for a wide spectrum of patients of different ages and subtypes of SMA (36, 37).

Clinical trials with Risdiplam

The FIREFISH trial is a continuing study that consists of two parts. It is an open-label multicenter research conducted in newborns that have SMA type 1 and 2 *SMN2* copies. Part 1, which has been finished, was an initial phase aimed at determining the appropriate dosage, assessing safety, and studying the pharmacokinetics and pharmacodynamics. A total of twenty-one babies, ranging in age from 1 to 7 months, and with a condition that started at a median age of 2 months, were included in the study. Initially, all participants had low baseline scores on the CHOP INTEND and HINE-2 evaluations, and none of them were able to sit with no help. During the trial period, most of the adverse events (AEs) encountered were mostly linked to decreased respiratory function resulting from the underlying disease, rather than the study medicine. These included respiratory tract infections, acute respiratory failure, and respiratory distress. Four individuals succumbed, all due to complications associated with SMA (38). The pharmacokinetic findings indicated a 2.1-fold increase in the concentration of SMN protein in the blood, compared to the initial level, four weeks after initiating the high-dose treatment. The provided data was used to establish the treatment dose of 0.2 mg per kilogram for the subsequent phase of the investigation. Further investigations on the effectiveness of risdiplam revealed a favorable clinical outcome. By the time they were 12 months old, 41% (7 out of 17) of the participants exhibited the capacity to sit alone for at least 5 seconds (38).

SUNFISH consists of two separate phases. The first phase, with 51 participants, focused on determining the appropriate dosage. The second phase assesses the efficacy and security of risdiplam in a heterogeneous population of individuals diagnosed with SMA types 2 and 3. Each segment of the research consisted of separate cohorts of patients, and part 2 is now in process. The findings from Part 1 demonstrated a significant improvement in motor performance in comparison to a control group that did not receive any kind of therapy. The research design for part 2 is a Phase 3 trial, which is randomized and double-blind (39). The study spans a duration of twelve months, during which patients are given either 2 doses of risdiplam or a placebo. This continues by an additional 12-month period when every participant receive risdiplam in an open-label fashion. Afterwards, all individuals will be eligible for a three-year open-label extension. The second phase of the research had a total of 180 participants, with 71% diagnosed with SMA type 2 and the remaining 29% diagnosed with SMA type 3. The participants ranged in age from 2 to 25 years and had limited mobility, being unable to walk. However, they were capable of sitting alone for a minimum of 5 seconds. Notably, there were no explicit criteria for eliminating persons with scoliosis, contractures, or those who needed nutritional or respiratory care (39).

Risks of Risdiplam

Like nusinersen and onasemnogene abeparvovec, most adverse effects (AEs), including substantial AEs, seem to be associated with the progression of the underlying disease or related complications, rather than the medicine itself. Consequently, persons with SMA type 1 have a higher frequency of serious AEs compared to those with types 2 or 3. The most often reported AEs linked to risdiplam in the FIREFISH and SUNFISH experiments are fever, diarrhea, mouth and aphthous ulcers, arthralgia, urinary tract infection, constipation, and skin rash. There have been instances of skin-related issues perhaps linked to risdiplam that have resolved on their own. Initial studies done on risdiplam in cynomolgus monkeys, which were exposed to high amounts of the drug, showed retinal impairment after being treated for a period of 5-6 months (39, 40).

SMA gene therapy challenges

The main difficulties related to gene therapy are the handling and delivery of the complex therapeutic material (AAV9 vector with transcription-competent SMN cDNA) and the effective distribution of the introduced gene throughout the body, including the central nervous system (CNS). Gene therapy raises additional issues about the overexpression of the introduced gene and the immune response triggered by the AAV9 vector. Neuronal tissues are very vulnerable to low levels of SMN in severe SMA. However, there is increasing data that suggests there are broad developmental abnormalities throughout the embryonic phases of severe SMA (41). It is premature to draw any conclusive judgments on the enduring impacts of gene therapy. Nevertheless, it is informative to consider a prior investigation involving non-human primates and piglets who received intravenous infusion of a modified AAV9 carrying the SMN gene, as well as more recent research done on mice with a severe form of SMA. These studies provide information on the potential long-term impacts of AAV9-based gene therapy for SMA. The mice research specifically identified synapse loss and motor neurodegeneration as a result of the toxicity caused by the overexpression of human SMN using AAV9-mediated gene therapy (42). The majority of individuals with SMA saw statistically significant gains from onasemnogene therapy. Significantly, those who had previously had nusinersen treatment also saw positive effects from gene therapy. Nevertheless, the results uncovered several treatment-induced adverse events. For example, pyrexia was the common adverse event seen in all three clinical studies with onasemnogene. Other negative occurrences seen

Table1. Names and frequencies of genes involved in the occurrence of lung cancer (2).

Drug	Type	Function	Clinical Trials	Status
Nusinersen (Spinraza)	Antisense oligonucleotide	Modifying splicing to enhance the creation of SMN protein by binding *SMN2* mRNA	ENDEAR (Phase III) NURTURE (Phase II) CHERISH (Phase III) DEVOTE (Phase II/III) ONWARD (Phase III)	Approved by FDA in 2016
Onasemnogene abeparvovec (Zolgensma)	Gene replacement therapy	Adenovirus vector (AAV9)-mediated delivery of the *SMN1* gene	START (Phase I) STR1VE (Phase III) NCT05089656 (Phase III)	Approved by FDA in 2019
Risdiplam (Evrysdi)	Small molecule	Directly binding to ESE2 of the *SMN2* transcript	FIREFISH (Phase II, III) SUNFISH (Phase II, III) JEWELFISH (Phase II) RAINBOWFISH (Phase II)	Approved by FDA in 2020
Olesoxime	Cholesterol-like compound	Reducing neuronal degeneration and death	Phase 2 OLEOS trial	Development stopped
Reldesemtiv **CK-2127107**	Fast skeletal muscle troponin activator (FSTA)	Increase muscle strength and cytokinetics	Phase 2 trial (NCT02644668)	Phase III trail is planned
SK-015	Monoclonal antibody	Promote muscle cells growth and division	TOPAZ (PhaseII)	Ongoing clinical trials
Pyridostigmine	Acetylcholine esterase inhibitor	Improved muscle strength and fatigue	SPACE Trial	Phase II

in younger children with SMA included bronchiolitis, pneumonia, respiratory distress, and respiratory syncytial virus bronchiolitis. Previous findings indicate that individuals treated with onasemnogene had significantly elevated levels of SMN expression in the liver, which may be attributed to the hepatotropism of the AAV9 vector (31, 43).

Although Zolgensma has shown favorable outcomes, there are still existing hazards. This medication has been approved for two specific groups of patients: those who have been diagnosed with 5q SMA and have a mutation in both copies of the *SMN1* gene, as well as a clinical manifestation of *SMA1*, and those who have been identified with 5q SMA and has a mutation in both copies of the *SMN1* gene, along with up to 3 copies of the *SMN2* gene. Currently, there is a lack of evidence addressing age or weight limits for these specific patient groups. In theory, a substantial population of SMA patients would have the potential to undergo Zolgensma therapy. Nevertheless, the existing clinical trial results only pertain to infants under six months old and weighing less than 8.4 kg. Limited information is accessible regarding the safety and efficacy of Zolgensma in older or heavier patients (44). A significant issue that might arise is a reduction or absence of treatment effectiveness owing to the existence of pre-existing antibodies against AAV9 in the community of individuals with SMA (45). Despite the confirmation of all the limits, a substantial issue will persist—the exorbitant cost of this medication, which amounts to almost $2 million for a single dosage of Zolgensma. It is among the most costly medications available for purchase (44).

Nucinersen is now the only licensed therapy medication for children and adults with SMA, a group of neurodegenerative illnesses. It shows promise as an ASO (antisense oligonucleotide) treatment for many neurodegenerative diseases. While the central nervous system has a long-lasting half-life, a significant disadvantage is the requirement of providing 4 initial doses and then three annual maintenance doses, resulting in patients having repeated intrathecal administrations. If the single genotype correction of Zolgensma does not achieve the expected outcomes, Spinraza treatment remains a feasible option for these patients (22). Another issue associated with medications

that control pre-mRNA splicing is the potential for unintended effects on non-target molecules or processes. Risdiplam and Branaplam have shown the ability to cause significant disruptions in the process of splicing, resulting in the inclusion of unintended exons, skipping of exons, retention of introns, elimination of introns, and the use of alternative splice sites. Hence, it is essential to discover more efficient dosage schedules to properly use existing therapeutic medicines that target splicing modulation (46).

CONCLUSIONS

SMA is a hereditary condition that leads to progressive muscle degeneration and loss of function due to issues with the *SMN1* gene. It is an autosomal recessive condition, meaning that both copies of the gene must be affected for the sickness to occur. In all patients, there is a second gene called *SMN2* located near the centromere. This gene is not damaged, but it does have a variant in exon 7 where a C nucleotide is replaced by a T nucleotide. This variation affects a splice enhancer, causing exon 7 to be excluded in most of the transcript produced by this gene. As a result, the protein produced by *SMN2* is unstable and cannot replace the mutant SMN1 protein. After conducting effective research on illness models and doing in-depth investigations on the roles of SMN during the last decade, it has been proposed that targeting SMN2 for overexpression might be a potential therapeutic strategy. Thanks to the introduction of three recently developed treatments, namely nusinersen (Spinraza), onasemnogene abeparvovec (Zolgensma), and risdiplam (Evrysdi), patients now have increased survival rates and better overall results. Nevertheless, patients and their families still encounter several obstacles related to the use of these treatments, such as inadequate treatment outcomes and a fluctuation in the advantages experienced by those who do react. This indicates that the pursuit of finding a cure for SMA is ongoing. In recent times, we have seen a significant milestone when the first medication that modifies diseases has obtained permission from the Food and Drug Administration. This treatment is now accessible to patients outside the confines of the clinical study. This medication is a novel antisense oligonucleotide that, when given by intrathecal administration, has the ability to enhance the inclusion of exon 7 in most of the *SMN2* mRNA and boost the creation of fully functional SMN protein. Despite facing significant criticism about its price, Zolgensma is a very successful single-dose therapy for SMA. It offers a higher cost-efficiency compared to similar medicines, Evrysdi and Spinraza, and necessitates shorter treatment duration. The concise characteristics of Zolgensma are very likely to contribute to the future popularity of this drug among doctors. Administering Zolgensma to children less than two years of age early on has been supported by significant research, showing superior results. Due to the effectiveness of this medication, clinicians may have more confidence in diagnosing SMA early, leading to improved survival rates and quality of life for their patients. Adverse effects linked to this gene therapy include of localized injection responses, feelings of nausea, increased levels of ALT (alanine aminotransferase), and hypersensitivity reactions. Newborn screening and prompt therapy with these innovative medications will soon be established as the customary approach for managing SMA. Nevertheless, it is important to note that the advantages of newborn screening and prompt intervention for SMA are only applicable to infants, and there are still several unsolved concerns for older children and adults who have SMA. Despite being first deemed incurable, the advent of novel treatments in the early 21st century has resulted in a tremendous improvement in the standard of life for those afflicted with SMA. When we contemplate the history of the SMA, we are amazed by the remarkable accomplishments of our predecessors and are motivated by the extraordinary scientists of our era. This essay aims to enhance the understanding of physicians and young researchers on the introduction of SMA illness and its treatment approaches.

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Conflict of Interest

Authors declared no conflict of interest.

Consent for publication

Not Applicable

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Evaluation of Severity Persistent Asthma with Hemophilus Influenza Infection in Asthmatic Patients

Emal Zoweiar Alsheihani[1], Ali Neamati[2*], Mohammad Reza Khakzad[3]

[1]MSc of Cellular & Molecular Biology, Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran.
[2]Associate Professor of Physiology, Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad Iran.
[3]Assistant of Immunology, Innovative Medical Research Center, Department of Immunology, Mashhad Branch, Islamic Azad University, Mashhad, Iran.

Corresponding author: Ali Neamati, Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad IranEmail: neamati.ali@gmail.com.

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Abstract:
Asthma is one of the most common non-communicable diseases characterized by reversible obstruction of airflow. It poses many problems for all age groups from infancy to old age. Various studies have shown that the occurrence of viral infections is associated with the severity of asthma symptoms so it can be prevented by controlling viral agents. In this study, the severity of the symptoms of persistent severe asthma with Haemophilus influenza infection was investigated. 31 patients with asthma with different degrees of disease were studied in this study. The results showed that in patients with asthma, the percentage of people with Haemophilus influenza was 71% and in 29% of other asthma cases, Haemophilus influenza virus was not observed. The relationship between asthma, cough and dyspnea with Haemophilus influenza infection showed that with increasing asthma symptoms, the severity of infection increases, and no significant association was observed between cough and dyspnea with Haemophilus influenza. Therefore, the results of this study clearly show that Haemophilus influenzae virus causes asthma symptoms to worsen in patients.

INTRODUCTION

The lung is the central organ of the respiratory system. On the other hand, in addition to receiving oxygen, the body must expel toxic gases from the body, this task is the responsibility of the lungs. Lungs are pink organs located in the chest. The right lung contains three lobes and the left lung has two lobes. Each lobe contains a curtain called the pleural membrane, which prevents friction between the lobes and makes them slide on top of each other. Lungs include lung tissue, air passages or bronchi and air sacs or alveoli. The inner wall of the trachea is covered with a slimy membrane with fine hairs or cilia that trap and remove dust particles. The trachea is divided into two trachea or tracheal tree and sacs and trachea. The respiratory system consists of two main parts, the upper respiratory system and the lower respiratory system. In addition to these organs, certain muscles of the chest play a role in breathing, the most important of which is the diaphragm, which is located under the lungs and separates the chest from the abdomen. Smaller muscles between the ribs are also involved in breathing (1-4). Respiratory disease, which is generally related to lung disease, includes a group of diseases that cause lung dysfunction by involving part or parts of the respiratory system. Lungs are the most important part of the respiratory system, which plays a role in the exchange of respiratory gases to supply oxygen to different tissues of the body and remove carbon dioxide. Every year, lung diseases affect many people in society, which reduces the level of performance of the person in daily activities. Respiratory diseases are the most common cause of referral to general practitioners in England. The degree of respiratory dysfunction in a lung disease depends on the type of disease and the extent of the damage. Lung diseases can be upper respiratory tract infections, pneumonia or chronic obstructive pulmonary disease. Chronic respiratory diseases are among the 10 life-threatening diseases in the world. According to the statistics of the World Health Organization, hundreds of millions of people in the world suffer from chronic respiratory diseases. It is expected to become the third cause of death in the world after cardiovascular diseases and cancers by 2030 (5-9).

MATERIALS AND METHODS

Collection of samples

In this study, 31 asthmatic patients and 31 controls were examined. Pulmonary tests such as FEV1, FENO, ACT were examined in the patients, and sputum samples were taken and sputum cytology was taken to determine the percentage of lymphocytes (Lym), the percentage of macrophages (MO), the percentage of neutrophils (Neu) and the percentage Eosinophil (EO) was investigated. Also, the severity of shortness of breath, severity of cough, stage of asthma and duration of asthma and clinical information including age, gender, height, weight and body mass index of patients were obtained from their files.

STATISTICAL ANALYSIS OF DATA

In the description of the data, appropriate statistical tables and indices such as the mean, etc. have been used, and in the data analysis, the normality of the data has been investigated using the Shapirovix test, which confirmed the normality of the method. Appropriate parametric tests such as Student's test and analysis of variance (Tukey's test in pairwise comparisons) were used, and the Mann-Whitney test was used for non-normal data. The chi-square test was used in the analysis of data with a nominal scale and in cases where more than 20% of the frequencies. Expected tables were less than 5 (Cochran), and Fisher's Exact Test was used. A linear model has been used for general analysis. The software used in this research is SPSS v.26 and the significance level of the tests is less than 5% (values less than 5% are marked with "*" in the results).

Haemophilus influenza virus detection method

A real-time one-step RT-PCR method manufactured by AllplexTM (Cat. No. RP9801X) was used to detect and measure the Haemophilus influenza virus. The reagents used are shown in Table 1.

The basis of the PCR method is shown in Table 2. RT-PCR Mastermix prepared according to Table 2. The contents of the solution prepared in Table 2 were vortexed for 5 minutes. One microliter of RT-PCR Mastermix was poured into the PCR tube and then 8 microliters of nucleic acid samples were added to it. By centrifuging the tube, the rest of the steps were performed according to the relevant instructions to detect Haemophilus influenzae.

RESULTS

Analysis of the parameters

Distribution of main variables

In this study, in the control group, 14 people (45.2 percent) were men and 17 people (54.8 percent) were women, and in the asthma group, there were 19 people (61.3 percent) men and 12 people (38.7 percent). There were women, and (chi-square test) no statistically significant difference was observed between the two groups (Likelihood Ratio=1.63, P=0.309).

As can be seen in Table 3, there was no statistically significant difference (Student's test) between the two groups for the parameters of age, weight, height, body mass index and body surface index (P>0.05). In this

Table 1. Reagents used in the RT-PCR method

Symbol	Contents	Volume	Description
PRIMER	5X PR1 MOM	500 µL	MuDT Oligo Mix (MOM): . Amplification and detection reagent
ENZYME	Real time One step Enzyme	200 µL	Enzyme mix for one step RT-PCR
BUFFER	5X Real time One step Buffer	500 µL	Buffer for one step RT-PCR -Buffer containing dNTPs
CONTROL +	RP1 PC	80 µL	Positive Control (PC): -Mixture of pathogen and IC clones
CONTROL IC	RP-V IC	1000 µL	Exogenous Internal Control (IC) for All plex Respiratory Panel 1,2 and 3
WATER	RNase-free Water	1000 µL	Ultrapure quality, PCR-grade Negative Control (NC): . Sterilized water as Negative Control

Table 2. Preparation of RT-PCR Mastermix

5X RP1 MOM	5 µL
RNase-free Water	5 µL
5X Real time One step Buffer	5 µL
Real time One step Enzyme	2 µL
Total volume of One step RT-PCR Mastermix	17 µL

Table 3. Demographic parameters

Variable	Test		Asthma		Control	
	Average	standard deviation	Average	standard deviation	Average	standard deviation
Age	P=0.079	t=-1.78	14.13	58.58	52.03	14.72
Weight	P=0.893	t=-0.135	10.67	73.19	72.84	10.03
Height	P=0.188	t=1.33	8.08	163.45	166.48	9.78
BMI	P=0.302	t=-1.04	4.00	27.49	26.44	3.99
Body surface area index	t=0.339	P=0.736	0.15	1.82	1.83	0.15

study, based on the body mass index in the control group, 10 people (32.2%) were normal, 14 people (45.2%) were overweight 7 people (22.6%) were obese, and 9 people were in asthma group. (29.0%) were normal, 12 (38.7%) were overweight and 10 (32.3%) were obese.

Distribution of asthma duration, asthma severity and symptoms

The mean and standard deviation (minimum and maximum) of the duration of asthma in the examined patients are 11.13 and 6.36 (3 and 25) years, respectively. Other information is shown in Figure 4.

Distribution of asthma indicators

As can be seen in Table 5, for all variables such as ACT.Score.pre, FEV1.pre, FENO.pre, Lym percentage, Mq percentage, Neu percentage and Eo percentage, there is a statistically significant difference (Student's test) between the two groups ($P<0.05$**). Other results in this study show that:

- According to the ACT.Score. pre-index, in the control group, 31 people (100.0 per cent) were normal, 0 people (0.0 per cent) were abnormal, and in the asthma group, 0 people (0.0 per cent) were normal, 31 (100.0%) were abnormal.
- According to the FEV1.pre-index, in the control group, 24 people (77.4 per cent) were normal, 7 people (62.2 per cent) were abnormal, and in the asthma group, 0 people (0.0 per cent) were normal, 31 people (100.0 per cent) were abnormal.
- According to the FENO.pre-index, in the control group, 31 people (100.0 per cent) were normal, 0 people (0.0 per cent) were abnormal, and in the asthma group, 2 people (6.4 per cent) were normal, 29 people (63.6 per cent) were abnormal.

Investigating the effect of different variables on Hemophilus influenza infection

A linear model has been used to investigate the effect of quantitative variables on Hemophilus influenza. At first, the variables of sex, age, body mass index, body surface index, asthma severity, asthma duration, and ACT.Score.pre, FEV1.pre, FENO.pre, mQ, neu and Eo were entered into the model (Table 6). Based on the obtained results, it was found that the severity of asthma has a significant relationship with Hemophilus influenza ($P<0.05$).

Relationship of different variables with Hemophilus influenza infection

This section will investigate the relationship of different parameters with Hemophilus influenza infection. Figures 1 to 4 examine the relationship between the severity of Hemophilus influenza infection and the parameters of asthma severity, ACT.Score, FEV.pre, FENO.pre, %neu and %EO.

DISCUSSION

Among the vast number of chronic diseases, asthma is one of the most common chronic disorders of the respiratory system, which has a significant prevalence and incidence. Asthma is an intermittent, reversible, and obstructive disease of the airways, which is characterized by the excessive response of the bronchi to various stimuli. These changes cause narrowing of the airways and shortness of breath in the patient, and it is one of the most common chronic diseases worldwide, with approximately 300 million people worldwide suffering from this disease. Of this amount, 10 to 12

Table 4. Distribution of severity and symptoms of asthma

Variable		Number	Per cent
Asthma severity	1	5	16.1%
	2	11	35.5%
	3	6	19.4%
	4	9	29.0%
Cough	1	4	12.9%
	2	7	22.6%
	3	17	54.8%
	4	3	9.7%
Shortness of breath	2	10	32.3%
	3	21	67.7%

Table 5. Asthma indices

Variable	Test		Asthma		Control	
	probability value	Test statistics	standard deviation	Average	standard deviation	Average
ACT.Score. pre	P=0.0001**	=24.69	2.12	11.03	1.40	22.29
FEV1.pre	P=0.0001**	=11.59	13.98	51.35	9.15	86.03
FENO.pre	P=0.0001**	=07.95	23.48	43.10	4.12	7.71
%Lym	P=0.0001**	=-4.69	8.54	21.26	3.92	13.32
%Mq	P=0.0001**	=45.94	5.55	22.45	5.00	81.77
%neu	P=0.0001**	=-25.82	9.92	36.45	1.69	3.74
%Eo	P=0.0001**	=-17.04	5.95	18.71	.18	1.03

percent are adults and it is expected that 100 million people will be added to the population of asthma patients in the world by 2025. In a report published in 2003, the prevalence of asthma in Iran in the entire population was estimated at 5%. This disease is classified into different types based on the severity of clinical symptoms, and all its forms are considered in this study. Asthma is a major problem in most parts of the world and its diagnosis and treatment is still a health problem. A large number of people suffering from this disease die every year. The death rate due to asthma is increasing in most countries. In Iran, according to the statistics reported by the Asthma and Allergy Clinic, 10% of Iranians have asthma. In the United States, one out of every 20 people has asthma, and 14 to 15 million people with asthma live in the United States. Asthma changes the family life and social activities of the sufferers and limits the physical activity of the patients, which leads to mental problems such as anxiety, depression and sadness in the patients. In this way, asthma affects various aspects of the patient's life (10-13). Bizintino and his colleagues investigated the relationship between rhinovirus C and the severity of asthma symptoms. In this study, 128 children with acute asthma in the age range of 2-16 years were investigated. The results of this study showed that children with chronic asthma and infected with rhinovirus C had more severe asthma attacks, so it can be concluded that rhinovirus C causes an increase in the severity of asthma symptoms(14).

Carroll and colleagues investigated the relationship between bronchitis and the risk of developing asthma in infants. In this study, 90,341 children were examined, 18% of whom had bronchitis. The results showed that children who were hospitalized with bronchiolitis during infancy, compared to children who did not have any symptoms of bronchiolitis, showed an increase in asthma in early childhood,

and this study confirms that diseases Pulmonary infections play an important role in the occurrence of asthma (15).Gerke and his colleagues investigated the relationship between seasonal influenza and the severity of asthma symptoms. The results of this study showed that based on time series regression models, there is a strong and significant relationship between the simultaneous activity of influenza and the incidence of asthma hospitalization. The results of this study confirmed that influenza activity is associated with an increase in the severity of asthma symptoms and these results show that improvement in the monitoring, analysis and prevention of influenza can reduce hospitalization in asthma patients (16).

CONCLUSION

Asthma is one of the common non-communicable diseases characterized by reversible airflow obstruction. It causes many problems for all age groups, from infancy to old age, and the people of Iran are not exempt from this. This disease can be controlled through simple education, and by using simple solutions, the suffering of these patients can be alleviated, finally, the country can be freed from spending huge costs in the field of acute asthma attacks and the absence of sufferers from work and social activities. Various studies have shown that the occurrence of viral infections is related to the severity of asthma symptoms, so its progress can be prevented by controlling viral factors. Therefore, in this study, the severity of persistent severe asthma symptoms with Haemophilus influenza infection was investigated. The results of this study showed that in patients with asthma, the percentage of people with

Table 6. Variables included in the model to investigate the severity of asthma with Haemophilus influenzae

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	2853.989[a]	12	237.832	1.831	.119
Intercept	36.873	1	36.873	.284	.601
Gender	9.757	1	9.757	.075	.787
Age	7.082	1	7.082	.055	.818
BMI	27.189	1	27.189	.209	.653
Body surface area index	.266	1	.266	.002	.964
ACT.Score.pre	12.361	1	12.361	.095	.761
FEV1.pre	12.492	1	12.492	.096	.760
FENO.pre	79.307	1	79.307	.611	.445
Lym	110.375	1	110.375	.850	.369
mQ	285.373	1	285.373	2.198	.156
Neu	219.391	1	219.391	1.689	.210
Eo	80.363	1	80.363	.619	.442
Asthma severity	1209.413	1	1209.413	9.313	.007
Error	2337.512	18	129.862		
Total	29670.535	31			
Corrected Total	5191.501	30			

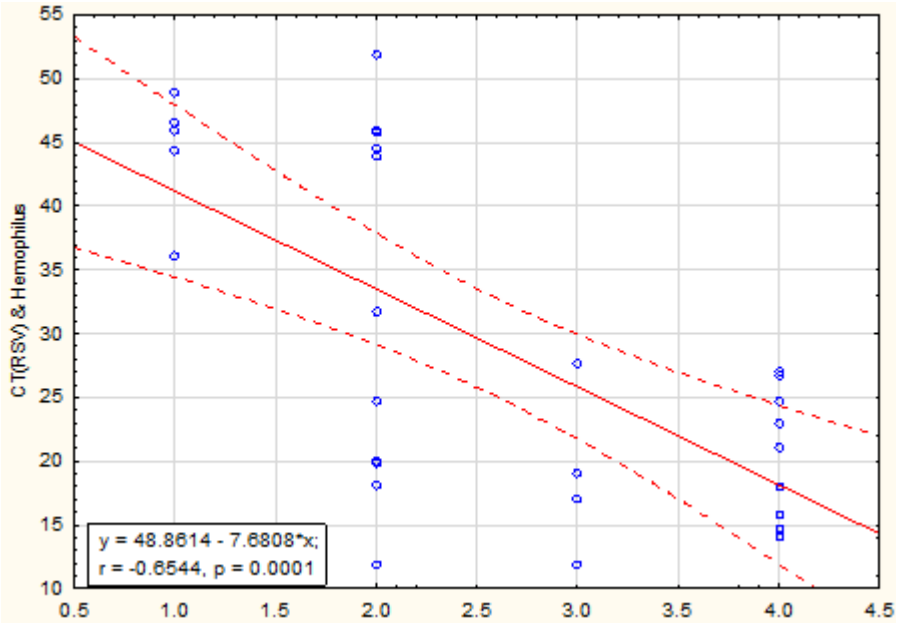

Fig 1. Correlation of asthma severity with Haemophilus influenza

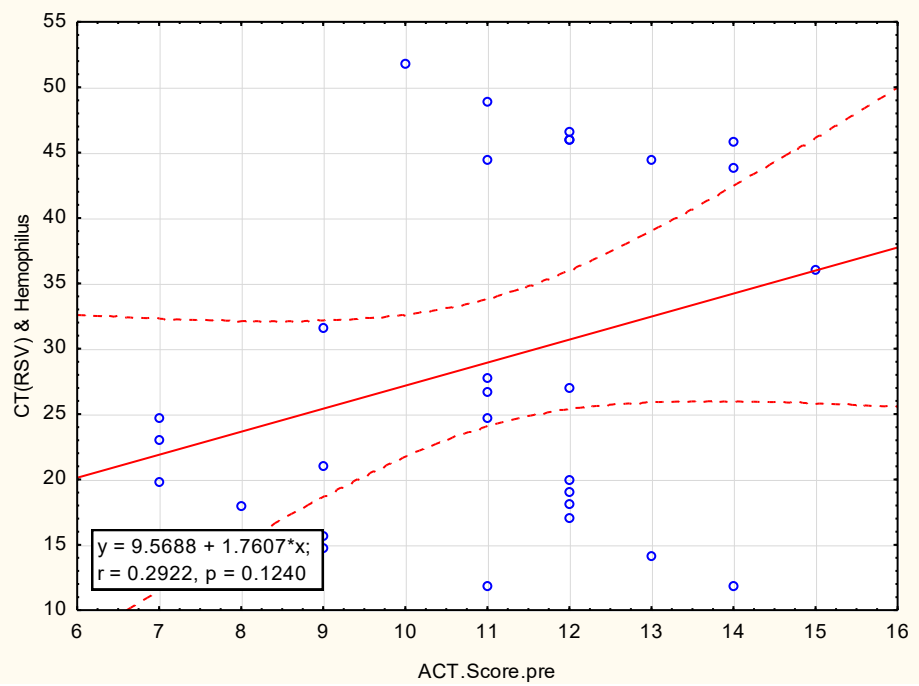

Fig 2. Correlation of ACT. Score pre with Haemophilus influenza

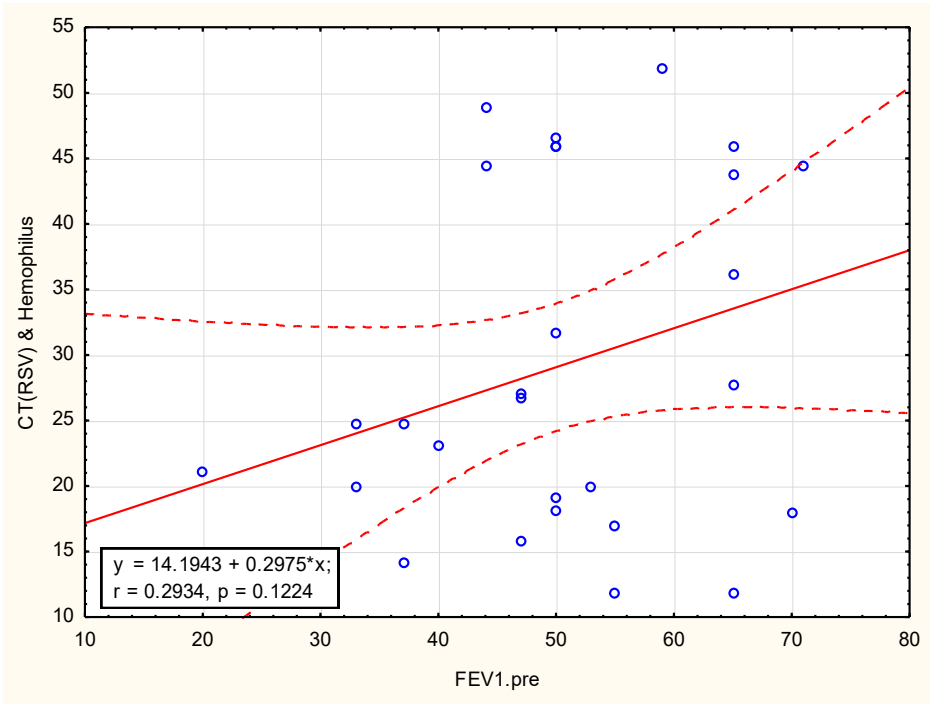

Fig3. FEV1.pre association with Haemophilus influenza

Haemophilus influenza is 71%, and Haemophilus influenzae virus was not observed in 29% of the remaining asthma cases. The relationship between the severity of asthma, cough and shortness of breath with Haemophilus influenza infection showed that with the increase in asthma symptoms, the severity of the infection increases, and no significant relationship between cough and shortness of breath with Haemophilus influenza was observed. Therefore, the results of this study clearly show that Haemophilus influenza virus causes worsening asthma symptoms in patients. Also, the relationship between asthma severity variables, ACT. Score, FEV.pre, FENO. pre, and %Eo with Haemophilus influenza infection were studied and the results showed that: There is a significant relationship between the severity of asthma symptoms and Haemophilus influenza infection (P =0.0001).

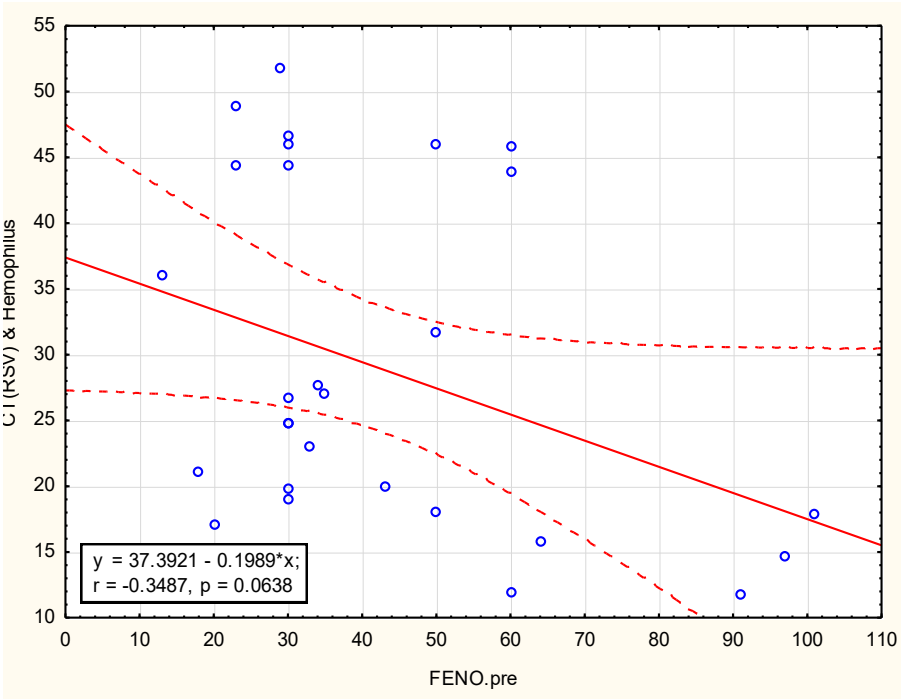

Fig4. FENO.pre association with Haemophilus influenza

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest.

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