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Experimed is glad to announce that the first issue of 2023 published nine original research articles and one valuable review to the literature belonging to 14 different universities from Turkiye.

I am very gratefully sending my deepest thanks to,

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Dear Institute Director Prof. Dr. Günnur Deniz who has expressed her supportive gratitude for Experimed to University's management platform,

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We are very pleased that our growing influence in the literature with this amazing Experimed Team gives us more courage to shed more light on science with new publications.

Prof. Dr. Bedia Çakmakoğlu

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REVIEW ARTICLE

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Delta Secretase and BDNF Signalling in Alzheimer's Disease

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ABSTRACT

As one of the major contributors of the central nervous system, neurons require neurotrophic factors, which are synthesized from neighbouring cells, for several cellular processes, such as neuronal survival, growth, and differentiation. Neurotrophic factors are categorized into the neurotrophin family, the neuropoietic cytokines, and the glial cell-derived neurotrophic factor. The neurotrophin family comprises four growth factors: nerve growth factor (NGF), neurotrophin-3 (NT3), neurotrophin-4 (NT4), and brain-derived neurotrophic factor (BDNF). One of the best-known neurotrophic factors is BDNF. Its importance is based on its central role in neuronal survival. Entry of the BDNF into the neurons occurs via TrkB receptors, and it is transported to the cell body along microtubules in axons. As it is known in the brains of Alzheimer's patients, the axonal transport of BDNF is destructed via the hyperphosphorylated tau. There are several causes for the hyperphosphorylation of tau. Among them, delta secretase (δ-secretase), a lysosomal cysteine protease, cleaves both amyloid precursor protein (APP) and tau. It is supposed to play an essential role in tau hyperphosphorylation, particularly in the aging brain. In this review, we focus on the activity of δ-secretase, how it leads to tau hyperphosphorylation, and how it disrupts the axonal transport of BDNF in Alzheimer's disease.

Keywords: BDNF, axonal transport, delta secretase, Alzheimer's disease, APP, tau protein

INTRODUCTION

Secretases Participate in Pathological Mechanisms of Alzheimer's Disease

Alzheimer's Disease is a degenerative brain disease and is one of the most common forms of dementia, causing 60-80% of all cases in the world (1). Dementia is a decline in cognitive ability that interferes with daily life activities (2). This decline occurs because of neuronal damage and destruction in the parts of the brain involved in cognitive functions (1). Studies to reveal the etiopathogenesis of Alzheimer's disease are continuing intensively, both in terms of the negative effects on the individual and his/her relatives and the socioeconomic effects of the disease. The incidence of age-related neurological diseases has increased due to the aging of the world population, and this disease has turned into a direct public health problem today.

As it is well-known, there are two possible hallmark pathologies for Alzheimer's Disease; extracellular amyloid plaques and intracellular neurofibrillary tangles (NFT). Firstly, amyloid plaques are pleated sheets of ss-amyloid peptide (3). Ss-amyloid is generated from the amyloid precursor protein (APP). In the physiological process, APP is cleaved by α and γ secretases. However, cleavage of APP due to the proteolytic action of β and γ secretases creates Ss-amyloid which is known mostly for its pathologic functions (4). Having been recently identified, the role of delta secretase (δ-secretase) on the molecular mechanism is still elusive (5). On the other hand, NFTs are composed of hyperphosphorylated tau protein (4). Tau is a cytoskeletal microtubule-associated protein that is regarded as a microtubule stabilizer (6). When hyperphosphorylated, tau proteins that normally bind to the microtubules leave the microtubule and form neurofibrillary tangles via clumping together (7). In 2014, Zhang et al. found that δ-secretase

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participates in the proteolysis of tau, as well (8). Furthermore, imbalanced distribution and abnormal regulation of the neurotrophic factors refer to one of the pathophysiological mechanisms underlying Alzheimer's disease. Neurotrophic factors have a feedback mechanism with δ-secretase, in which disorganisation destructs the balance and gives rise to the neurodegenerative disease (9).

Brain-Derived Neurotrophic Factor (BDNF) Takes Essential Neural Processes

Neurotrophic factors play an essential role in the proliferation, differentiation, growth, and survival of nerve cells as endogenous proteins and act as crucial ligands. In this way, they perform the functions throughout the developmental stages (10). Besides their role in neurodevelopment and several other neural processes, they are also involved in maintaining neural plasticity in the central and peripheral nervous system (11). According to the structure, target, and signalling pathways, neurotrophic factors are categorized into the neurotrophin family, the neuropoietic cytokines, and the glial cell-derived neurotrophic factor (12). The neurotrophin family comprises four growth factors: Nerve growth factor (NGF), neurotrophin-3 (NT3), neurotrophin-4 (NT4), and BDNF (10). Following the discovery of the nerve growth factor in the early 1950s by Rita Levi-Montalcini, BDNF was first purified from a pig brain in 1982 (13). Proteolytic cleavage of neurotrophins is required to transform them from an immature synthesized form to a mature form (14). The distinct neurotrophins act via two receptors: Tyrosine-related kinase (Trk) receptors and p75 neurotrophin receptor (p75NTR) (11). BDNF and TrkB receptors were the main focus of studies on neurodegenerative and neuropsychiatric disorders in the last decades. BDNF activity is present in the basal forebrain, cortex, and hippocampus. The localisation also implicates the roles of BDNF in thinking, memory formation, learning, and synaptic plasticity (15). Alterations in expression and the activity of BDNF are reported in many neurodegenerative and neuropsychiatric disorders (16). Studies based on animal models have revealed the alteration of expression levels of BDNF in neuropsychiatric and neurodegenerative disorders (17).

Regulation of BDNF and its Ligand Binding Required for Normal Processing of Nervous Systems

The human *BDNF* gene is located on chromosome 11p14.1, and encodes the BDNF protein (11). Synthesis of BDNF occurs in the soma of neurons and glia and is transported to nerve terminals (18). Precursor protein BDNF (pre-pro-BDNF) is the first synthesized form of BDNF in the endoplasmic reticulum (ER), where the pre-pro-BDNF is folded, and its proteolytic

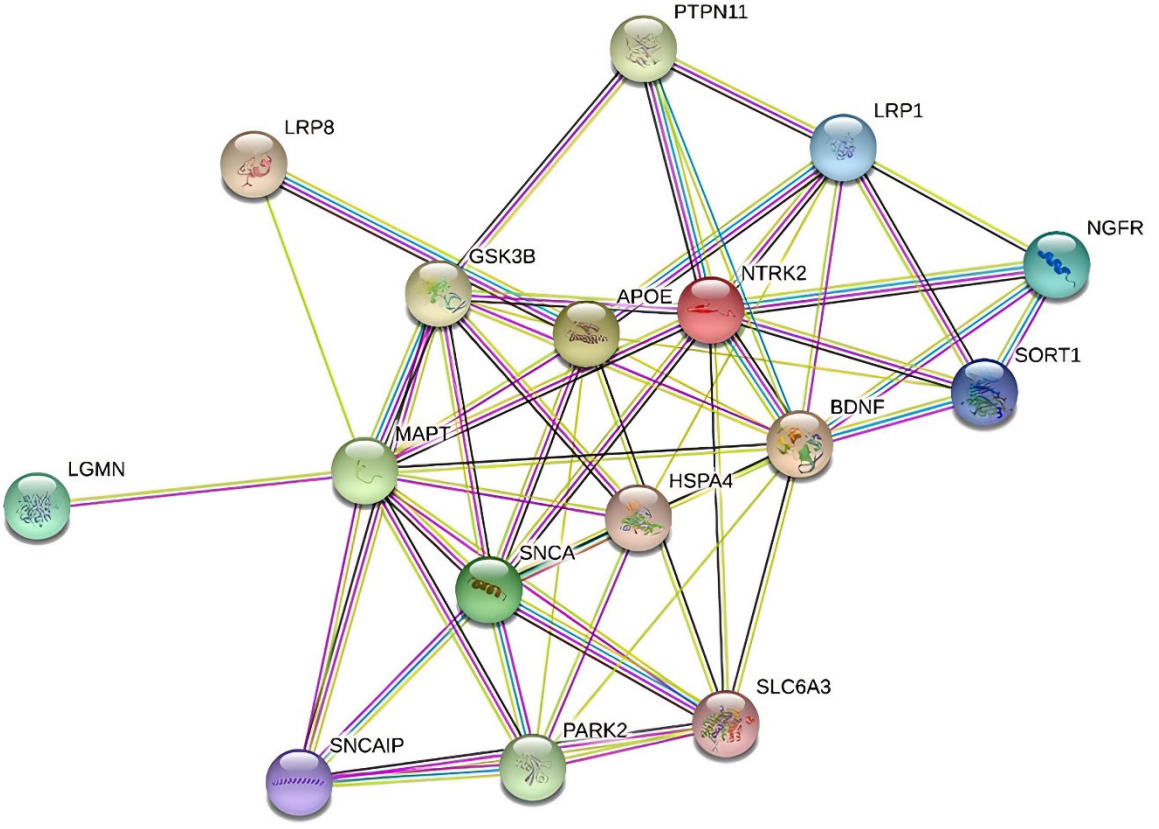

Figure 1. The direct interaction between LGMN (δ-secretase) and MAPT (microtubule associated protein tau), and its indirect interactivity with synuclein alpha (SNCA), apolipoprotein E (ApoE) as shown on the network (STRING).

cleavage takes place to give rise to the protein precursor form of BDNF (pro-BDNF) (32 kDa) (19). Further proteolytic cleavage of the pro-BDNF consists of either intracellular or extracellular cleavage. The constitutive pathway of the trans-Golgi network involves the intracellular proteolytic cleavage of pro-BDNF, the liberation of furin, the packaging of mature BDNF (mBDNF) into vesicles, and the fusing of the vesicles with plasma membrane (11). On the other hand, the plasmin system and matrix metalloproteases 2 and 9 (MMP2 and MMP9) are involved in the extracellular processing of pro-BDNF, which can act as endogenous ligands directly (20). Influx of Ca^{2+} via N-Methyl-D-aspartate (NMDA) receptors, which is permeable to Ca^{2+}, and voltage-gated Ca^{2+} channels take part in the neural activity based on the regulation of transcription of BDNF (21).

Extracellular pro-BDNF and mBDNF exert distinct physiological responses (19). So, maintaining the proper ratio of pro-BDNF to mBDNF is critical during different neurodevelopmental stages. BDNF binds to two different types of receptors with a distinct affinity. The first receptor is p75NTR, a transmembrane-spanning protein called nerve growth factor receptor (NGFR). It is a member of the tumour necrosis factor receptor (TNFR) superfamily (22). It possesses structurally various domains, including a carboxy-terminal intracellular domain with a flexible juxta membrane adaptor protein-binding region, amino-terminal extracellular domain, and globular death domain (22). Regulation of receptor conformation and ligand binding is mediated by four cysteine-rich domains in the p75 amino-terminal extracellular domain (23).

Another receptor of BDNF is tropomyosin-related kinase B (TrkB), which is one of the Trk receptor families responsible for regulating synaptic strength and plasticity in the adult nervous system (13). The BDNF-TrkB signalling pathway plays a role in various neuronal diseases. The overexpression of BDNF is also suggested to be related to pathological conditions (24). BDNF/TrkB signalling is critical for neural development, survival, differentiation, and plasticity; it is involved in transcription, translation, and protein trafficking, which occur during phases of synaptic development (25). The binding of BDNF to TrkB triggers activation of the downstream mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (PLCγ) pathways (26). The MAPK and PI3K play a critical role in the translation and trafficking of proteins induced by synaptic activity. The PLCγ is responsible for the regulation of intracellular Ca^{2+} that is

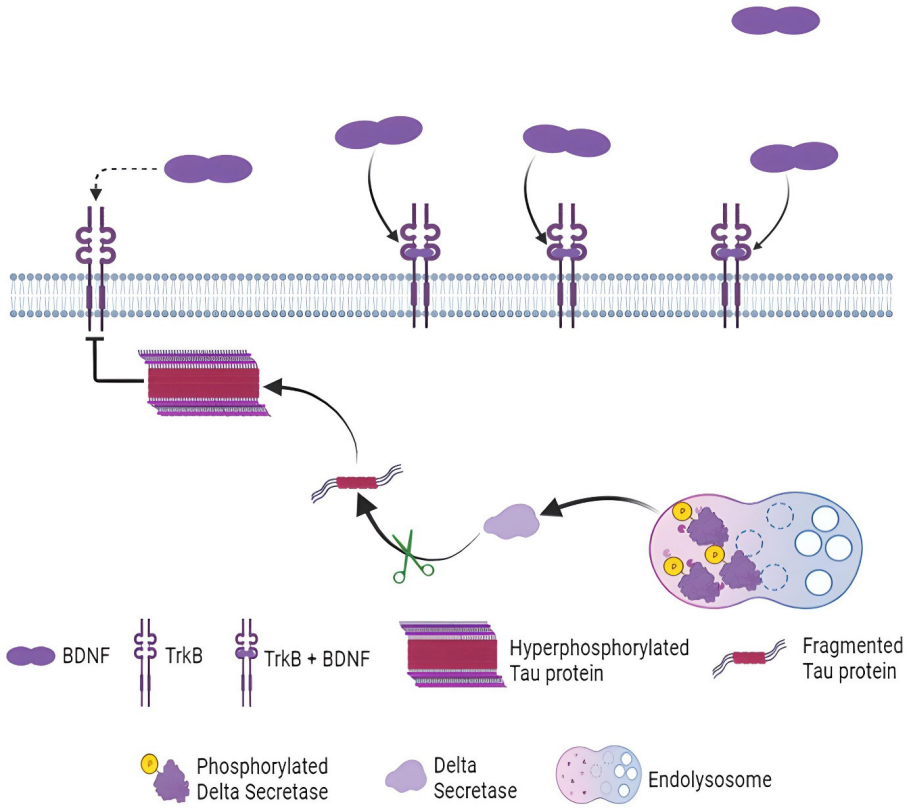

Figure 2. Blockage of the TrkB receptor by hyperphosphorylation of tau via the action of activated delta secretase. Delta secretase is located in the endolysosome as an inactive form. Decreasing the BDNF level lowered the phosphorylation of delta secretase and resulted in the activation of delta secretase. Activated delta secretase cleaves the tau protein. The specific tau fragment is sensitive for phosphorylation. The formed tau fragment tends to bind the TrkB receptor and gives rise to blockage of BDNF binding to the TrkB receptor. BDNF: Brain-derived neurotrophic factor, TrkB: Tyrosine kinase B (drawn by using BioRender).

involved in transcription via cAMP and protein kinase C (25). The deficiency of BDNF/TrkB signalling is the underlying reason for the neurodegeneration in AD. The release of BDNF to the extracellular space can be acute and/or gradual, so different cellular mechanisms can be activated. The acute increase of BDNF concentration in extracellular space reveals transient activation of the TrkB receptor, which enables dendritic growth and spine morphogenesis. A gradual increase of BDNF concentration in extracellular space reveals sustained activation of the TrkB receptor, which initiates dendritic arborization and spinogenesis (26).

δ-secretase Cleaves APP and Tau

δ-secretase is called asparagine endopeptidase (AEP, also known as Legumain - LGMN), a cysteine proteinase, and plays an important role in cleavage after specific asparagine residue (5). In humans, the *LGMN* gene, which is located on chromosome 14q32.12, encodes δ-secretase (27). The primary location of δ-secretase is endolysosomes (28). Synthesis of δ-secretase occurs as an inactive proenzyme, so it is cleaved in its N- and C-terminal propeptides to be activated under acidic conditions (28). Autocatalytic cleavage of δ-secretase is necessary to transform zymogen pro-δ-secretase, 56 kDa, into its active form (29). Post-translational modifications at distinct pH values drive catalytic activity of δ-secretase. So, the regulation of enzymatic activity of δ-secretase is based on pH. Aging leads to upregulation of δ-secretase in the brain and causes simultaneous cleavage of APP, specifically at N373 and N585 residues and tau (5). Aging also raises the activity of δ-secretase and tau cleavage (8). Furthermore, the S226 residue of the δ-secretase is phosphorylated by a cell cycle kinase called SRPK2 by which phosphorylation of δ-secretase at S226 residue takes place and in turn leads to its translocation into cytoplasm (30). Cleavage of BACE at N294 is carried out by δ-secretase, which accelerates its proteolytic activity and elevates amyloid pathology (31). The BACE N294 fragment that is formed raises the δ-secretase activity (31).

The specific cleavage of APP increases Aβ production and enables senile plaque formation. Cleavage of APP by δ-secretase after N585 residue gives rise to the formation of 586-695 APP fragments, and β- and γ-secretase easily cleaves the fragments to yield Aβ (27). As the cleavage of APP by δ-secretase occurs after N373, it yields the toxic 1-373 APP fragment (27). When the AEP cleavage of APP is blocked, amyloid deposition and production are reduced (5). In addition, hyperphosphorylation of tau arises in Alzheimer's disease (32). 1-368 tau fragment arises from cleavage of tau after N368 by δ-secretase (27). The tau fragment is more susceptible to phosphorylation in comparison with full-length tau (27). Formation of tau fragment leads to disturbance of microtubule assembly activity of tau (8). NFT formation by hyperphosphorylated tau results in Alzheimer's disease pathology. The brain of human wild-type APP/tau transgenic mice was injected by δ-secretase, which facilitates the process of senile plaques and NFT formation in both genders, results in both synaptic and cognitive defects (33). Absence of the δ-secretase activity resulted in the sharp decrease of NFT pathology and recovery of cognitive functions, as shown by P301S mice (33). Moreover, binding 1-368 tau fragment to TrkB causes blockage of neurotrophic signals and induction of neuronal cell death (9).

BDNF Phosphorylates δ-secretase

Phosphorylation of δ-secretase by BDNF-activated Akt on T322 residues regulates its lysosomal translocation and inactivation (34). A decrease in BDNF levels causes weakened δ-secretase phosphorylation, which activates it, and its translocation into cytoplasm arises (35). On the other hand, tau, especially the δ-secretase–truncated tau N368 fragment, specifically binds to TrkB receptors, an interaction that BDNF antagonizes. Tau N368 strongly interacts with the TrkB receptor C-terminal tail, a site of PLC-γ1 binding. This action is down-regulated by the presence of BDNF (36). Binding of tau N368 fragment to TrkB receptors evokes blockage of neurotrophic signals, which triggers cell death (34). BDNF or TrkB receptors knockout gives rise to declined phosphorylation of δ-secretase on T322 residue, contributes to cleavage of tau 368 residue, occurrence of Alzheimer's disease pathology and cognitive abnormalities (9).

Transcription factor C/EBPβ plays an important role in the age-dependent augmentation of δ-secretase activity in the brain (37). Upregulation of C/EBPβ is correlated to deficiency of BDNF/TrkB, which exacerbates inflammatory cytokines and triggers the JAK2/STAT3 pathway. Upraised δ-secretase expression and fragmentations of APP and tau take place in return (34). BDNF-provoked Akt phosphorylation of δ-secretase at T322 residue results in its inactivation and it resumes locating in lysosome (35). Blockage of BDNF neurotrophic signals is via cleavage of TrkB receptor at N365 and N486/489 residues on the extracellular and intracellular domain (ICD), respectively, by δ-secretase (38). Depletion of C/EBPβ led to inhibition of the expressions of APP, tau and δ-secretase, and restrained APP and tau cleavage, and resulted in alleviation of Alzheimer's disease pathology from 3xTg mice (33). Alzheimer's disease pathology was diminished in δ-secretase knockout 3xTg mice (33). As has been proven, C/EBPβ manages Alzheimer's disease pathology by affecting the δ-secretase activity. Depletion of BDNF in primary neuronal culture induces BDNF/TrkB signalling and increases inflammatory cytokines, stimulates the JAK2/STAT3 pathway and transcription factor C/EBPβ, and results in high δ-secretase expression (33).

δ-secretase Cleaves Inhibitor-2 Protein Phosphatase-2A (I2PP2A) and Results in Abnormal Hyperphosphorylation of Tau

Under acidic conditions, another protein cleaved by δ-secretase is inhibitor-2 protein phosphatase-2A (I_2^{PP2A}) at Asn-175 neuronal cytoplasm, which produces I_{2NTF} and I_{2CTF} (39, 40). I_2^{PP2A} is a SET protein, is also called template-activating factor (TAF1β), which is found in the neuronal cytoplasm and is also an inhibitor of PP2A (41). The level of I_2^{PP2A} in the brains of Alzheimer's Disease patients is higher than in normal brains (42). PP2A accounts for ~70% of tau protein phosphatase activity in the adult human

brain (39). Inhibition of PP2A phosphatase can change the balance between phosphorylation and dephosphorylation of tau protein, so results in increasing tau phosphorylation (40). Inhibition of PP2A activity occurs due to the interaction of the N- and C- terminal fragments of I_2^{PP2A}, which are cleavage products I_{2NTF} and I_{2CTF} (39, 40). Translocation of I_2^{PP2A} from the neuronal nucleus to the cytoplasm with the co-localization of PP2A results in abnormal hyperphosphorylation of tau by the inhibiting of PP2A phosphatase in the brains of Alzheimer's disease patients due to δ-secretase (39).

Activity of δ-secretase Might Engage Axonal Degeneration and Prompt Dislocation of BDNF

A variety of substances are shuttled bidirectionally throughout the axon microtubule of neurons. The ATP-dependent process is called axonal transport (43). The kinesin superfamily of motor proteins takes part in transporting substances in anterograde transport (44). In addition, cytoplasmic dynein participates in retrograde transport. Retrograde axonal transport plays a vital role in essential processes such as neurotrophic factor signalling, autophagy, and lysosomal degradation (43). Axonal transport provides intracellular trafficking over a long distance which is highly regulated to supply the normal function of neurons and cell viability (43).

Tau plays an essential role in the function of normal axonal transport (45). The binding capacity of tau and its ability to stabilize microtubules declines with the phosphorylation of tau (46). Glycogen synthase kinase-3 (GSK-3) is considered to be the primary kinase of phosphorylation of tau (32). Augmented tau phosphorylation slows down tau transport in neurons, and impeding tau phosphorylation by GSK-3 decreases its motion (47). The importance of tau is connected to its major role in the normal axonal transport mechanism (45). Tau effectuates the outcome of Aβ on axonal transport with an unknown mechanism (45). Aβ-induced malfunction in axonal transport is blocked by dwindling endogenous tau (48). So, it is assumed that δ-secretase might participate in axonal degeneration by interacting with tau (Figure 1).

The activation of TrkB on axon terminals by BDNF gives rise to the stimulation of signal pathways connected to neuronal survival (45). BDNF-TrkB complex emergence is followed by their endocytosis from the plasma membrane, and microtubules in the axons mediate their retrograde transport (49). Impairment of BDNF-mediated TrkB axonal transport occurs in Alzheimer's disease transgenic mouse neurons (50). Although the exact mechanism underlying axonal transport of the BDNF-TrkB still remains mysterious, it is claimed that Aβ oligomers deteriorate retrograde transport of BDNF (45). So, the effect of δ-secretase on the axonal transport of the BDNF-TrkB may be based on its role in Aβ pathology (Figure 2).

CONCLUSION

In this review, we discussed the involvement of δ-secretase in altered BDNF/TrkB signal mechanisms, its role in the pathophysiology of Alzheimer's disease. In addition, we discussed the possible contribution of BDNF downregulation to the deterioration of Alzheimer's disease. As detailed above, in BDNF downregulation, there are many connected processes, including the activity of δ-secretase and the hindering activity of the trophic factor by blockage of the TrkB receptor. Thus, even though the characterization of the relationship between BDNF and disease symptoms is undeniably challenging on account of the multiple processes which regulate the amount of BDNF in tissues, elaborate mechanisms underlying the downregulation and deteriorated axonal transport of BDNF by the activity of δ-secretase should be elucidated to provide more potent therapeutic approaches. In addition, understanding the function of δ-secretase in Alzheimer's disease pathology, the association between the destruction of axonal transport of BDNF and the role of δ-secretase in tau hyperphosphorylation might unravel one part of the molecular mechanism of Alzheimer's disease pathology, in addition to other neurodegenerative and neuropsychiatric disorders. Contributing to axonal degeneration, δ-secretase supports BDNF dislocation, while BDNF has the potential to phosphorylate δ-secretase, which is necessary for its lysosomal translocation. The elusive direct and indirect roles of δ-secretase in the pathological mechanism of neurodegenerative diseases, in particular Alzheimer's disease, may be revealed through further research.

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Histopathological and Serum Biomarkers Analyses in MRONJ due to Periodontal Disease in Rats: Comparison of Zoledronic Acid and Denosumab

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ABSTRACT

Objective: This study aimed to investigate the bisphosphonate and denosumab effects in medication-related osteonecrosis of the jaws (MRONJ) caused by periodontal disease with analyses of serum biomarkers and histopathology.

Materials and Methods: Forty Copenhagen rats were used in the study. A ligature wire was wrapped around the first molars to induce periodontal disease. The rats were divided into a zoledronic acid group (ZG) (n=12), a denosumab group (DG) (n=12), a saline group (SG) (n=10), and a control group (CG) (n=6). Prostate cancer was induced by injections for ZG, DG, and SG following the ligature application, and injections were repeated on the 14th and 21st days. While periodontal disease was evaluated clinically with gingival edema, swelling and redness, serum osteocalcin, osteopontin, parathormone and receptor activator of nuclear factor-kappa B ligand (RANKL) levels were evaluated using the LUMINEX technique. The Mann-Whitney U test was used for the comparison of parameters between groups ($p<0.05$).

Results: The osteocalcin levels were increased in CG, RANKL levels were decreased in DG, osteopontin levels were increased in ZG, and parathormone levels were increased in both ZG and CG.

Conclusion: Since the long-term use of bisphosphonates can cause osteonecrosis in the jaw bones, it should not be overlooked that this can also be caused by chronic inflammatory conditions such as periodontal disease.

Keywords: Bisphosphonates, denosumab, osteonecrosis, periodontal diseases, rats

INTRODUCTION

Periodontal disease is a common chronic inflammatory condition that affects about 20-50% of the global population (1). It is known that the etiological factor is bacteria, affecting the surrounding tissues of the teeth. The mildest form of periodontal disease is gingivitis, an infection only affecting the gingiva which is completely reversible with proper treatment. Bacterial dysbiosis increases when gingivitis is not treated and turns into periodontitis, which is an irreversible infection characterized by the destruction of epithelial attachment and alveolar bone (2).

The relationship between periodontitis and systemic diseases is increasing with the reports published day by day. The periodontal disease which is a globally common disease is suspicious in medication-related osteonecrosis of the jaws (MRONJ) etiology. It is also regarded as a risk

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factor for osteoradionecrosis of the jaws in the head and neck irradiated patients (3).

Prostate cancer is a well-known cancer type with a high rate of metastasis to the bone. The bone metastasis rate in prostate cancer is 74% (4). Bisphosphonates are used to reduce osteoclastic activity caused by androgen-deprivation therapy in bone metastasis of prostate cancer (5). Bisphosphonates are drugs that are frequently used to suppress the activity of bone metastasis and treat hypercalcemia which is a common complication of bone metastasis (6). Bisphosphonates which are pyrophosphate analogues have been used for a long time in the treatment of various diseases (5). Bisphosphonates bind with a high affinity to hydroxyapatites in the resorption areas of bones (7). Hence, they suppress osteoclast activity by reducing the development and aggregation of osteoclast progenitor cells (8).

As is well known, the use of bisphosphonates can lead to osteonecrosis of the jawbones (ONJ). This clinical condition was defined in 2003 and was named as Bisphosphonate-Induced Osteonecrosis of the Jaws (BRONJ) (9). Since then, a large number of patients showing serious consequences of these medication complications have been reported at a high growth rate (10). The American Association of Oral and Maxillofacial Surgeons (AAOMS) recommended a denomination from BRONJ to MRONJ in 2014 in order to accommodate the increasing number of ONJ cases in patients receiving other drugs such as Denosumab (Dmab), sunitinib, or rituximab (11, 12).

Dmab is a monoclonal human antibody (IgG_2) that is used in the treatment of bone metastasis (13). It affects osteoclastic activity by inhibiting osteoclast formation, reduces bone destruction by binding receptor activator of nuclear factor-kappa B ligand (RANKL) (14).

The etiopathology of osteonecrosis induced by bisphosphonates has been subjected to many studies in the past two decades (9). Dental invasive procedures and also infections such as periodontal diseases were the subjects of these studies, however, the reports on the occurrence of spontaneous osteonecrosis revealed another face of this question (15).

There are several proteins involved in bone metabolism, although some are more effective to monitor osteoblast activity and the activity of the drug used (16). These proteins are osteocalcin (OCN), which is synthesized by osteoblasts and is a marker of osteoblastic activity (17), osteopontin (OPN), which plays a role in binding osteoclasts to the mineralized bone matrix (18), and parathormone (PTH), which directly affects calcium metabolism and bone remodeling (19).

According to the literature, prostate cancer is one of the cancer types that most frequently causes bone metastasis (20), so a prostate cancer model in rats was created to examine the effect of periodontitis on MRONJ in oncology patients. This study aimed to investigate the effects of Dmab and bisphosphonate in MRONJ caused by periodontal disease with analyses of serum biomarkers and histopathology.

MATERIALS AND METHODS

This project has been reviewed and approved by the the Istanbul University Local Ethics Committee for Animal Experiments (2013/30). The study was carried out at Istanbul University, Aziz Sancar Institute of Experimental Medicine, Departments of Laboratory Animals Science and Molecular Medicine, and Istanbul University, Institute of Oncology, Department of Tumor Pathology.

Experimental Animals

Forty male Copenhagen rats, in the range of 270-300 gr body-weight, were obtained from Istanbul University, Aziz Sancar Institute of Experimental Medicine, Department of Laboratory Animals Science. They were accommodated in steel cages at an ambient temperature of 22±2 °C with 12 hours of light and dark cycles per day and had free access to fresh water and food ad libitum.

Experimental Procedure

General anesthesia was administered using 100 mg/kg ketamine HCl (50 mg/ml Ketalar®, Pfizer, United Kingdom) and Xylazine HCl (Rompun flakon, Bayer, Turkiye) injection. 0.25 mm-sized round retainer ligature wire was wrapped around the upper right first molars and fixed to the mesial of the tooth of all the rats to induce the experimental periodontal disease (Figure 1).

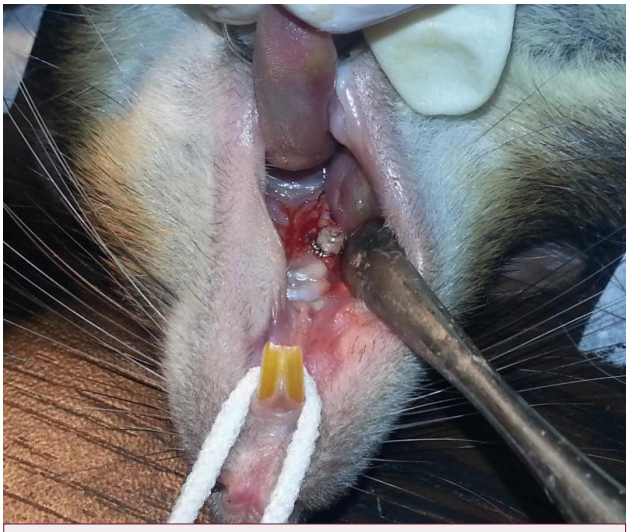

Figure 1. Placement of ligatures around 1st maxillary molar of rats.

Experimental Groups

Experimental animals were divided into four randomized groups: A zoledronic acid (ZA) group (ZG) (n=12), a Dmab group (DG) (n=12), a saline group (SG) (n = 10) and a control group (CG) (n=6).

Experimental Design

One week after the ligature application, prostate cancer was induced by the tumor cell line (R-3327MATLyLu) to the left

ventricle of the prostate of rats. Ten days after prostate cancer induction, the ZG received a weekly subcutaneous injection of 10 mg/kg ZA, the DG received a subcutaneous injection of 7.5 µg/kg of Dmab once a week, a weekly subcutaneous saline injection in the SG. All injections were repeated on days 7, 14 and 21 (21, 22). No intervention was made to the CG. All animals were clinically evaluated weekly regarding periodontal disease.

One week after the last injection, all animals were sacrificed with cervical dislocation. All tissues were dissected and evaluated before the histopathologic examination. For the periodontal disease examination, evaluation criterias were the presence of oedema, swelling and hyperemia at the gingiva. The maxilla and prostate of the experimental animals were then surgically removed for histopathological analysis. In addition, blood samples of 1 ml per animal from the cardiac tissue were collected and centrifuged at 1500 g for 10 min, and serum stored at -80°C until the immunoassays were conducted.

Histopathological Analysis

The samples were stained with the hematoxylin-eosin method and examined under a light microscope. The evaluation criteria under the light microscope (Olympus BX60 microscope, Tokyo, Japan) were determined as the presence of inflammation and necrosis in soft tissue and bone. Necrosis and foreign body reaction were graded as 0: no sign, +: existing. Inflammation was scored as 0 (absent), 1 (mild), 2 (moderate) and 3 (severe) (23). Prostate materials were stained with the hematoxylin-eosin method and examined under the light microscope to confirm tumor presence in each sample.

Immunoassay Method

Serum RANKL, OCN, OPN and PTH levels were determined using the LUMINEX technique according to the kits' instructions (MilliporeSigma, Merck KGaA, Darmstadt, Germany). 200 µl assay buffer was added to each well on the plate. The seal was placed on the plate and mixed with a plate shaker for 10 minutes. 25 µl of the appropriate matrix solution was added to the background, standard and control wells. The plate was sealed and covered with foil. It was incubated at 4°C for 16-18 hours in a plate shaker. The contents of the plate were poured out, the plate was washed 3 times. 50 µl detection antibody was added to each well. The plate was sealed and covered with foil. It was incubated for 1 hour on the shaker. Streptavidin-Phycoerythrin (50 µl) was added to each well containing 50 µl detection antibody. The plate was sealed and covered with the foil. It was incubated in the shaker for 30 minutes. The contents of the plate were poured out and the plate was washed 3 times. 100 µl of Sheath Fluid was added to each well. The beads were resuspended in the plate shaker for 5 minutes. The plate was run with the appropriate device (Luminex Corporation, Texas, USA) and Luminex IS 100 Software.

Power Analysis

The power analysis of the study was calculated using the G*Power 3.1.9.2 program. In the study in which 4 parameters were examined in 4 experimental groups, the minimum sample value was 10, a total of 40 rats were found in each group with a two-way alternative hypothesis, a medium effect size of 0.25, a power of 80% and a margin of error of 5%. In order to increase the number of rats in the experimental groups, it was decided to have 12 rats each in the ZG and DG groups, and 6 rats each in the control and serum groups.

Statistical Analysis

Statistical analysis was performed with SPSS 25.0. The conformity of the data to the normal distribution was evaluated with the Kolmogorov-Smirnov test. While the homogeneity of variance was evaluated with the Levene test, the Welch ANOVA test was used to compare the inhomogeneous variance measurements between the groups, and the Dunnett's T3 test was used as the post hoc test for statistically significant measurements. In comparisons between groups, the level of serum RANKL, OCN, OPN and PTH levels were measured with the Mann-Whitney U test. The statistical significance limit was accepted as $p<0.05$.

RESULTS

LUMINEX Analysis

During the experiment, 6 animals from ZG, 6 animals from DG and 6 animals from SG were lost due to cancer. Serum RANKL, OCN, OPN, and PTH levels are shown in Table 1. In terms of OCN levels, the difference between the groups was not significant. RANKL levels were statistically lower in the DG compared to the CG (p=0.032). OPN levels were statistically increased in ZG

Table 1. Comparison of serum levels of RANKL, OCN, OPN and PTH in study groups.

Groups	RANKL (pg/ml)	OCN (pg/ml)	PTH (pg/ml)	OPN (pg/ml)
CG (n=6)	10.37±8.26*	4466.23±3043.62	32.74±28.07°	293.60±69.61†
SG (n=4)	4.56±2.07	6727.82±667.91	1.66±1.28β,°	683.88±61.65
ZG (n=6)	9.97±6.04	6253.30±869.86	63.90±84.94β	733.73±126.43†,α
DG (n=6)	3.81±2.43*	5253.65±1023.82	15.83±11.82	321.91±237.35α

*: p=0.032, °: p=0.019, α: p=0.013, β: p=0.010, †: p=0.04, RANKL: receptor activator of nuclear factor-kappa B ligand, OCN: osteocalcin, PTH: parathormone, OPN: osteopontin, CG: control group, SG: saline group, ZG: zoledronic acid group, DG: denosumab group, pg/ml: picograms per milliliters

compared to the DG (p=0.013), and CG (p=0.04). PTH levels were increased in both the ZG (p=0.010), and the CG (p=0.019) compared to the SG.

In the clinical examination, edema, swelling and hyperemia, which were the symptoms of the periodontal disease, were observed in all ligatured tissues.

Histopathological Results

In the histopathological evaluation, in one of the experimental animals in the ZG, resorption, and local necrosis, and dense colonies of microorganisms were observed in the alveolar bone (Figure 2). In the soft tissue, chronic inflammatory infiltration including mainly lymphoplasmacytic infiltration, and a slight increase in the number of cells in the basal layer of the surface epithelium was observed. Numerous lymphocyte infiltrations within the epithelium were also noted. Regarding this experimental animal, the inflammation score was 3 and the necrosis was labelled '+'. Sections taken from all other experimental animals showed similar histopathological characteristics that no necrosis was seen and scored as '0' and inflammation scored as '1'. In all these sections, alveolar bone generally preserved its natural structure. A mild chronic inflammatory infiltration (score 1) was observed in the gingival pocket through which the ligature passed (Figure 3). A well-differentiated carcinoma was observed in the prostate of all animals except for the control group confirmed with

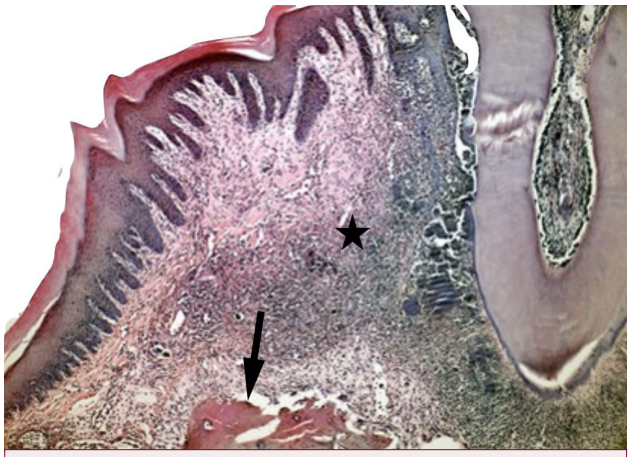

Figure 2. Severe chronic inflammatory infiltration (star) and alveolar bone loss (arrow) was observed under the surface mucosa demonstrating parakeratosis in the tissue sample obtained from the animal in ZG (H&E x 100).

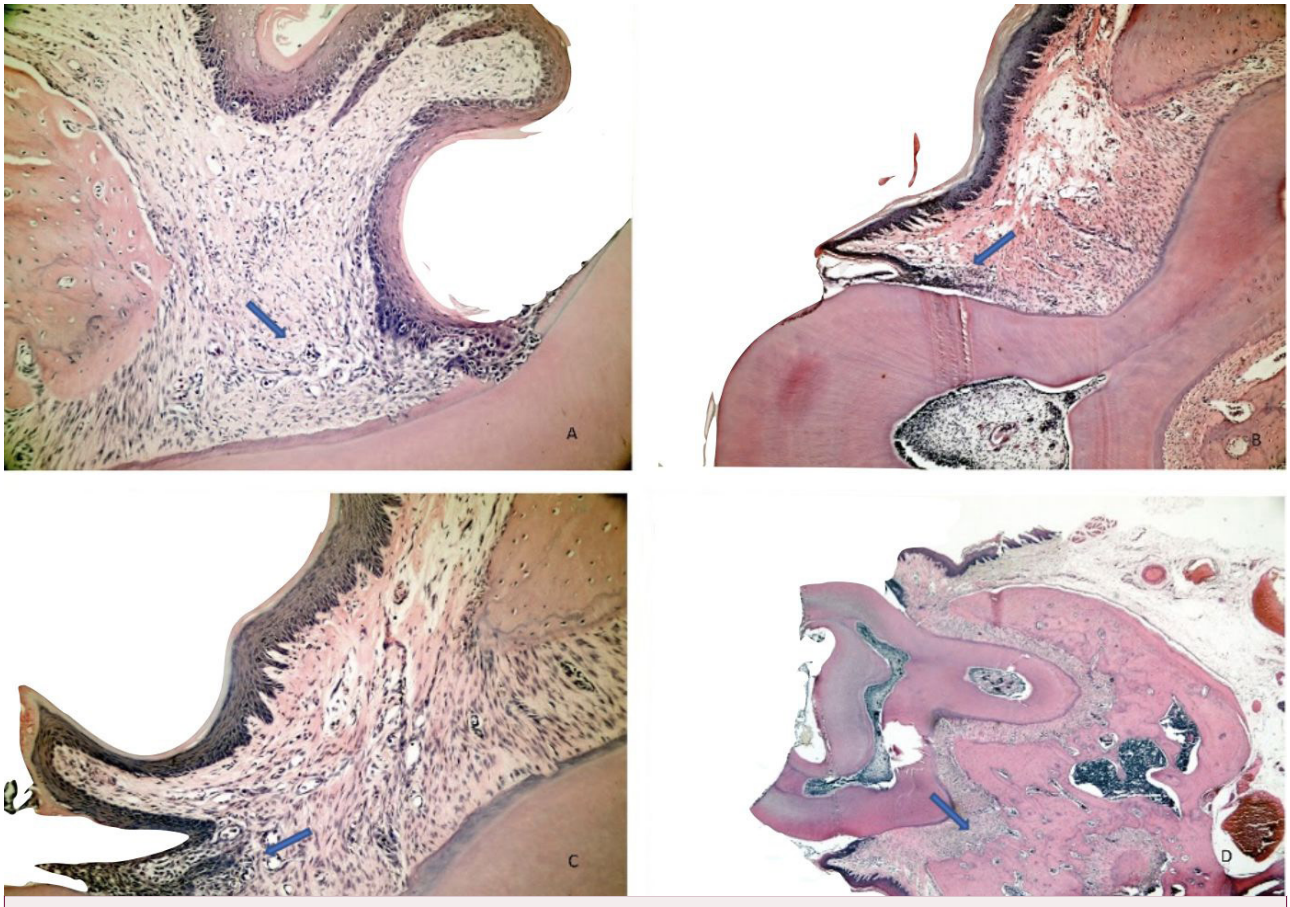

Figure 3. Representative histopathological images of the ZG (A), DG (B), SG (C), and CD (D) without any bone destruction, but with mild inflammation in the connective tissue (arrows).

histopathological examination. Also, distant organ metastasis developed in all animals except the control group. Metastases occurred most frequently in the lungs and the pancreas.

DISCUSSION

Prostate cancer is one of the most common malignancies among men, and bone metastases are the major cause of its morbidity and mortality. Rat models are frequently used in experimental studies on prostate biology, as the rat prostate has many common features with the human prostate and rats are one of the few species with spontaneous prostate adenocarcinoma (24). Male rats were used in the study to investigate the effects of ZA and Dmab hormone-independent, as estrogen secretion differentiates bone metabolism by affecting hormonal activity (25). Dunning R-3327 MATLyLu is an anaplastic, androgen-independent tumor cell line that can metastasize spontaneously, is an ideal drug for immunotherapeutic research in the treatment of metastatic prostate cancer (26). Therefore, Copenhagen male rats and Dunning R-3327MATLyLu tumor cell line were used in the study.

The tumor cell line can be injected into rats to induce prostate cancer by the subcutaneous, intravenous, intraosseous or orthotopic method (27). Since the survival time of the experimental animals in intravenous, intraosseous or subcutaneous applications of this cell line was reported as 15-20 days (28), the orthotopic method in which the cell line was directly injected into the prostate was used, and was reported as 30-35 days (29). Despite following a more conservative approach and daily subcutaneous saline injection, 18 rats were lost by the 28th day due to dehydration.

Since the characteristics of the molar teeth such as shallow gingival grooves and the attachment of the junction epithelium of rats are similar to those of humans, it is the most commonly used animal model to induce the experimental periodontal disease. Placing a ligature around the mandibular first molar tooth is a postulated, highly preferable model to induce experimental periodontal diseases in rats (29). Biofilm accumulation is increased around the ligature which was placed subgingivally and inflammation of periodontium is observed within an average of 21 days in this model (30).

Periodontal disease is a chronic inflammatory condition that develops as a result of host response, even though its etiological factor is bacteria. The diagnosis of periodontal disease, which is one of the most common chronic inflammatory diseases in the world affecting around 20-50% of the global population, can sometimes be overlooked even by dentists who focus on infection foci (1). Hence, the relationship between chronic periodontal inflammation and MRONJ, but not acute trauma such as tooth extraction, was evaluated in the study. There are several studies in the literature evaluating the relationship between periodontal disease and ZA, and more severe bone resorption was observed in the experimental group (31). However, there is no study evaluating the effect of Dmab on periodontal disease. The study is the first to examine the effect of not only ZA but also Dmab on osteonecrosis in chronic periodontal inflammation by evaluating OPN, OCN, PTH and RANKL levels.

Periodontal disease is a complex and multifactorial chronic disease that is characterized by the destruction of periodontal tissues, and eventually results in the loss of the tooth. Since OCN is a marker that is synthesized by osteoblasts and indicates bone formation, a decrease in the serum level of this protein is considered a disorder in bone metabolism (32). Related to this, the resorption of alveolar bone in periodontal disease also causes a decrease in the serum OCN level. Similarly, studies are reporting that OCN levels increase with periodontal treatment (33).

Likewise, studies are reporting that the OPN level, which is one of the important markers in the alveolar bone resorption process, is associated with periodontal disease (34). Some studies have shown that the OPN level increased proportionally with the progression of the disease, and it was significantly reduced with the periodontal treatment (35).

PTH is both an anabolic and catabolic hormone that regulates the calcium and phosphate mechanism of bone (19). There are many studies in the literature regarding the therapeutic effect of PTH analogues (36). Intermittent or continuous administrations of PTH are similar to the anabolic and catabolic effects of endogenous PTH secretion (37). Since the number of osteoblasts increases with the anabolic effect of PTH, it is used as a therapeutic agent in periodontal treatment. In addition, the serum level of PTH decreases in periodontal diseases (38). Accordingly, the PTH level is one of the parameters to be evaluated in periodontal infections.

The OCN level is a parameter that indicates osteoblastic activity, and functions of osteoblasts. Although it was found at the highest level in CG induced by periodontal disease, which is chronic inflammation, its low levels in other groups (ZG, DG, SG) indicate that osteoblastic activity is suppressed in these groups. Although there are a few studies in the literature that are proportional to this result (17), there are also studies reporting that the relationship between OCN level, and bisphosphonate is dose-dependent (39).

Dmab inhibits osteoclast formation, function and bone remodeling by suppressing the RANK-RANKL interaction (40). The highest level of RANKL was found in the control group. This is an expected result because there is no agent to suppress RANKL activity in the environment. Statistically, it is lower in DG compared to CG. When the RANKL results were examined, although a decrease was seen in the ZG, it was not as effective as the substance Dmab.

OPN contributes to homeostasis, remodeling, biomineralization, and wound healing by stimulating osteoclasts to bind to the bone matrix. OPN level is significantly higher in ZG than in DG. The levels of the other groups relative to each other are not significant. The high level of OPN is strong evidence for

the resorptive activity of osteoclasts (41). OPN levels were determined as CG<DG<SG<ZG, respectively. Bone necrosis and resorption were observed only in ZG animals from our study groups. When we compared the ZG and DG groups in terms of OPN levels, a significant increase in ZG was found. While a few studies are showing that bisphosphonates increase OPN levels, there are also studies showing that they decrease OPN levels (17,42). This difference may be due to the dosage, the duration, and whether intravenous (IV) or oral bisphosphonates are used. We recommend that there may be an increase in OPN levels in ZG to suppress the necrotic formations seen in animals in this group.

PTH regulates bone calcium metabolism, and remodeling by affecting osteoblasts and osteoclasts (19). There are studies in which PTH is used in the treatment of various tumors (43), and osteoporosis (44). Likewise, in a study evaluating bone loss, it was found to be significantly lower in the study group in which ZA, and PTH were used together (45). Similarly, the PTH level was found to be significantly lower in ZG and CG compared to SG in our study.

The loss of about one-third of the rats due to metastases of prostate cancer, and osteonecrosis, and periodontal disease-dependent bone resorption were found in only one animal constitute the limitations of our study. For further studies, researchers should consider rat models with prostate cancer for short-termed clinical studies since life expectancy could be shorter than expected, and could affect the results of the study.

In this study, in which alveolar bone resorption was created with periodontal disease, and drugs that suppress bone resorption were used, 4 parameters showing different functions were examined. Parameters such as OCN and PTH, which inhibit bone resorption, decrease in periodontal disease, and increase with periodontal treatment, were compared with parameters such as RANKL and OPN, which have antagonist effects, and the effects of tumor (hence bone remodeling) inhibitory agents such as zoledronic acid, and Dmab on these parameters were shown. The effect mechanism of periodontal diseases, which can be seen frequently in the clinic, in patients using bisphosphonate and Dmab, has been demonstrated regarding biomarkers and histologically.

CONCLUSION

Increased PTH and OPN levels, and decreased OCN levels may indicate that patients who have been administered ZA are more susceptible to osteonecrosis, especially in chronic inflammatory conditions such as periodontal diseases. The investigation of zoledronic acid and Dmab in different dental conditions in randomized controlled trials will provide a better understanding of osteonecrosis.

Ethics Committee Approval: The experimental procedures were approved by the Istanbul University Local Ethics Committee for Animal Experiments (Decision No. 2013/30).

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study - C.D.C., K.B.K., H.A.E., M.S.T.; Data Acquisition - M.S.T., H.A.E., R.B.K.Ü.; Data Analysis/Interpretation - M.S.T., H.A.E., F.Ç., Ü.Z., M.Ü.; Drafting Manuscript - R.B.K.Ü., K.B.K., M.S.T.; Critical Revision of Manuscript - R.B.K.Ü., K.B.K., Ü.Z.; Final Approval and Accountability - R.B.K.U., K.B.K.

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Investigation of Galectin-3 Levels of Endometriosis Patients According to Stages

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ABSTRACT

Objective: Endometriosis is a gynecological disease associated with chronic pelvic inflammation, pain, and infertility. Galectin-3 (Gal-3) is a protein that can bind to β-galactosides, which plays an important role in different biological processes according to the stages of the disease in patients with endometriosis. This study aimed to elucidate the importance of Gal-3 in endometriosis, to reveal its potential power as a non-invasive diagnostic biomarker in disease etiopathogenesis.

Materials and Methods: The serum concentration of Gal-3 and cancer antigen 125 (CA-125) from whole blood were measured using enzyme-linked immuno sorbent assay (ELISA) and an auto-analyzer, respectively. Gal-3 expression was determined by quantitative real-time polymerase chain reaction (qRT-PCR) from peripheral blood.

Results: We found significant differences for Gal-3 expression levels between the endometriosis and control groups ($p < 0.05$). Gal-3 levels in the serum of women with endometriosis are also remarkably increased compared with the control group.

Conclusion: Galectin-3 can play critical functions in the development and progression of endometriosis, so, further studies are needed in this area.

Keywords: Galectin-3, endometriosis , biomarkers, qRT-PCR

INTRODUCTION

Endometriosis is defined by the presence of endometrial glands and stroma outside the uterus, which is associated with pelvic pain, inflammation, and infertility in young women (1,2). The multifactorial etiopathogenesis of endometriosis includes environmental, genetic, epigenetic, endocrine or immune factors (3-6). Although the pathophysiology of the disease is not fully understood, there is increasing evidence of chronic dysregulation of inflammatory and vascular signaling in endometriosis (7). Patients often complain of dysmenorrhea, dyspareunia, pelvic pain and infertility, all of which lead to a reduced quality of life (8). Endometriosis is considered histologically a benign disease. But, development is similar to malignancy with regard to infiltration and attachment properties to other tissues (9, 10). It has been stated that endometriosis is mostly associated with ovarian cancer among all neoplasms. Many risk factors such as early menarche, short menstrual cycle duration and low parity are common for ovarian

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cancer and endometriosis (11). Diagnosis of endometriosis is delayed due to the complexity of the pathogenesis and the variety of symptoms (12). Laparoscopy is still the only reliable "gold standard" for diagnosing endometriosis. Laparoscopy is a surgical procedure with potential risks, and can be uncomfortable and sometimes even painful for the patient. From a clinical point of view, the development of a non-invasive test is important in the early diagnosis of endometriosis. Many molecules included in the pathogenesis of endometriosis have been examined as potential biomarkers. Nevertheless, they are not in the desired sensitivity and originality (13, 14). One of the most researched sources of biomarkers is peripheral blood (15). Peripheral blood is an important source of biomarkers because it is readily available and minimally invasive (16). Much research has been done on blood-based biomarkers for endometriosis (17). In recent years, lectins have become an important topic for reproductive immunology, inflammation and endometriosis (18, 19). Galectins (Gal) are glycan-binding proteins and are found in almost all organisms (20). Galectin-3 (Gal-3) has different roles in many biological processes; cell embryogenesis, adhesion, differentiation and proliferation. Although endometriosis is primarily an endometrioid type ovarian cancer, it can turn into many gynecological cancers (21). Gal-3 is predominantly found in the cytoplasm and secreted into biological fluids including serum and urine (22). Noel et al. reported that Gal-3 is increased in peritoneal endometriosis, different lesions, and eutopic endometrium. These data suggest that Gal-3 can promote the development or progression of endometriosis (18).

The high expression of Gal-3 in endometriosis suggests that these molecules could be potential diagnostic biomarkers for endometriosis and/or targets of new therapeutic approaches (23).

This study aimed to report Gal-3 expression levels in the stages of endometriosis patients, and to reveal their potential power as a non-invasive diagnostic biomarker.

MATERIALS AND METHODS

Study Design and Patients

In this study, the patient group consisted of 50 female patients who were pre-diagnosed with endometriosis (based on radiological and biopsy and histopathological results) in the Sivas Cumhuriyet University, Faculty of Medicine, Research and Practice Hospital, Gynecology and Obstetrics Clinic.

Seventy healthy volunteers without any pre-diagnosis were included in this study. The patients between the ages of 15-45 were selected and included in the study. Before the study, approval was obtained from the Ethics Committee of Cumhuriyet University, Faculty of Medicine (2019-10/06).

Approximately 5 ml of venous blood from each right or left forearm of 50 women diagnosed with endometriosis and 70 healthy women were collected into dry tubes. Patients that may affect cancer antigen 125 (CA-125) levels and mimic endometriosis as a history of rheumatic disease, chronic inflammatory disease or an autoimmune disease were not included in the study.

The study was carried out on the whole blood and EDTA containing peripheral blood serum samples taken from the patients. The whole blood samples were centrifuged at 3000 rpm for 10 minutes. Serum samples were stored at -80°C until the biochemical analysis. The remainder of the whole blood samples were separated to prepare total RNA and cDNA for Gal-3 expression by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) analysis.

Determination of CA-125 Levels

CA-125 levels, one of the best known and most widely used biomarkers for endometriosis, were measured using autoanalyzer (Roche Cobas e802, USA).

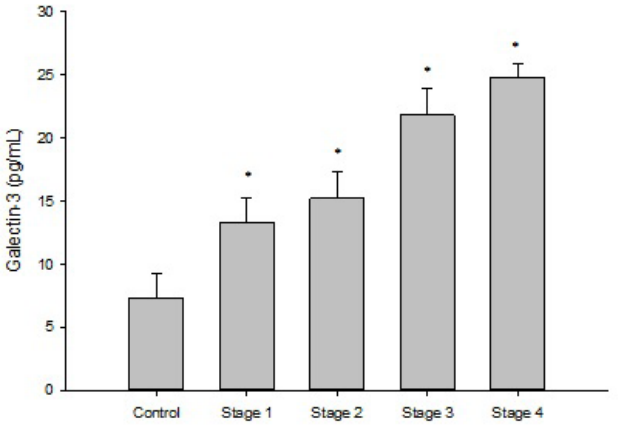

Figure 1. Results of Gal-3 levels in serum by ELISA. *: p<0.05, compared to the control.

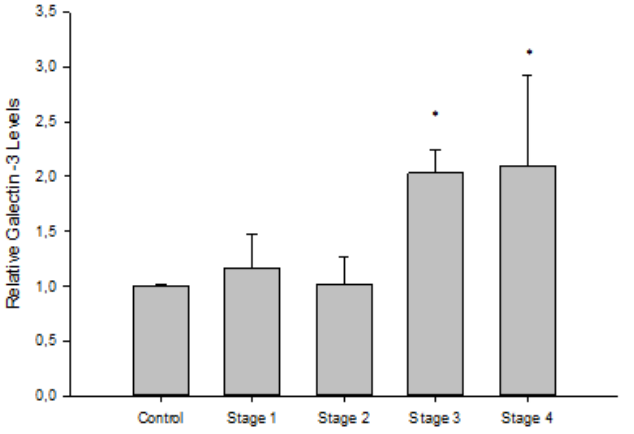

Figure 2. Results of qRT-PCR analysis Gal-3 levels between endometriosis and control group. *: p<0.05, compared to the control.

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qRT-PCR Analysis

The total RNA was extracted from whole blood collected by using the RNA Isolation Kit (GeneAll, Cat no:106-101, Korea) according to the manufacturer's recommendations. cDNA synthesis using a HyperScript cDNA synthesis kit (GeneAll Cat no: 601-710, Korea), according to manufacturer's protocols. Reaction mixtures were incubated at 25°C, 10 min; 55°C, 60 min; and 85°C, 5 min. cDNAs were measured using a qubit ssDNA Assay Kit (Molecular probes, Life Technologies, Cat No: Q10212, United States).

RT-qPCR was performed using Realamp sybr green master mix with high-ROX dye (Cat no:801-051, Korea), according to manufacturer's protocols. About 20µL PCR reaction included 4µL RT product, 1 µL (10pm) forward primer, 1 µL (10pm) reverse primer, 1µL ROX, 3 µL sterile water, and 10 µL (2X) SYBR master mix. Forward and reversed primers of Gal-3 are shown in Table 1. Gal-3 expression levels were normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample. The PCR reaction mixtures were incubated in at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 40 s. Relative increases in mRNA expression were processed using the $2^{-\Delta\Delta CT}$ method (24).

Determination of Gal-3 Levels by ELISA Assay

Serum Gal-3 levels were analyzed by an ELISA assay, according to the manufacturer's recommendations (Elabscience, Cat No: E-EL-H1470,USA).

Statistical Analysis

The data of this study were analyzed with the statistical program SPSS-22 (SPSS INC., Chicago, IL, USA). When α= 0.05, β= 0.10, (1- β)= 0.90 (R: Sample Allection Ratio:1.7) values were taken for this study, it was decided to include 50 patients and 70 non-patients in the study. The power of the test was determined as p=0.90064. The dependent variable of the study is endometriosis disease. Its main independent variable is Gal-3 level. Data indicated by measurement are presented descriptively with mean and standard deviation (minimum - maximum), data indicated by counting, number and percentage distribution. Student t test was used to compare the mean age of both groups, and the chi-square test was used for the analysis of nominal values. When the parametric test assumptions were fulfilled (Kolmogorof Smirnov), the significance test of the difference between the two means in independent groups was used. $p < 0.05$ was considered as statistically significant.

Table 1. Forward and Reverse Primers of Gal-3 expression.

Primers	5′ → 3′
Galectin-3	AGCCAACGAGCGGAAAATG (F)
	GCACTTGGCTGTCCAGAAGA (R)
GAPDH	GTCAAGGCTGAGAACGGGAA (F)
	AAATGAGCCCCAGCCTTCTC (R)

F: Forward; R: Reverse; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

RESULTS

The demographic baseline characteristics of the patient's examined groups are given in Table 2. The only variable between the patient and control groups was the surgical stages. The endometriosis with patients who underwent laparoscopy and laparotomy were divided into endometriosis stages, that are shown in Table 3. The patients were surgically divided into stages considering the Revised American Fertility Society criteria. According to the demographic data, the patients' ages, family histories and the presence of chronic pain had no statistically significant difference. CA-125 levels were found to be statistically significant in the endometriosis and control groups ($p < 0.001$). The presence of a history of infertility, whether there was a history of pregnancy in the past, menstrual pain, and pain during sexual intercourse were found to be statistically significant between the patients and controls (Table 2). As shown in Figure 1, the Gal-3 values of the endometriosis group are approximately 13.26 ± 1.96 pg / L for stage 1, 15.18±2.08 pg / L for stage 2, 21.79±2.13 pg / L for stage 3 and stage 4 as 24.79±1.07 pg / L, while the Gal-3 value of the control group was found to be 7.29±1.98 pg / L ($p < 0.05$, for all).

The results of the qRT-PCR analysis, no significant difference was found for Gal-3 levels between endometriosis and control group in stage 1 and 2. We found significant differences in Gal-3 expression levels among endometriosis with stages 3 and 4, and the control group ($p < 0.05$, for both) (Figure 2).

DISCUSSION

Endometriosis is an important gynecological disease associated with chronic pain and infertility, affecting the quality of life. It is known that many factors such as environmental, genetic, epigenetic, endocrine or immune factors are involved in the etiopathogenesis of endometriosis (3, 5). Although it is a comprehensive field of study, there are no reliable, specific biomarkers of endometriosis (25). Therefore, a novel biomarker with high specificity and sensitivity is needed for the diagnosis of endometriosis (26). Early diagnosis is significant for the early treatment of endometriosis and helps improve quality of life and preserve fertility (25).

Gals are a family of galactoside binding proteins that plays a role in many physiological and pathological processes, such as regulation of the immune system, cell growth, and angiogenesis (14, 23). High expression levels of Gals in patients with endometriosis and associated inflammation suggest that these molecules can be used as potential diagnostic biomarkers (23). Accumulating evidence from the literature shows that Gal-3 could be used as a diagnostic or prognostic biomarker for heart disease, kidney disease, and cancer (22, 27, 28). In recent years, lectins have become important in research on the immunity of the female reproductive system, pregnancy and infertility (5,14).

Table 2. Clinical characteristics in patient and control groups.

		Patient	Control	p-value
Ages[α]	(years, mean±SD)	38.36±9.667	36.41±13.41	0.383
		n (%)	**n (%)**	**p-value**
Family history[β]	-	40 (80)	64 (91.4)	0.101
	First degree relative	9 (18)	4 (5.71)	
	Distant relative	1(2)	2 (2.89)	
Infertility[β]	+	12 (24)	7 (10)	0.038*
	-	38 (76)	63 (90)	
Past pregnancy history[β]	+	12 (24)	5 (7.14)	0.050*
	-	0 (0.0)	2 (2.85)	
Painful menstruation[β]	+	38 (76)	34 (48.6)	0.002*
	-	12 (24)	36 (51.4)	
Dyspareunia[β]	+	23 (46)	47 (67.1)	0.038*
	-	3 (6)	3 (4.4)	
Chronic pelvic pain[β]	+	24 (48)	26 (37.2)	0.234
	-	26 (52)	44 (62.8)	
CA-125 levels[β]	Low	19 (38)	54 (77.1)	0.001*
	High	31 (62)	16 (22.9)	

+: Yes; -: No; n: number; *: $p < 0.05$, compared to control group.
α; Student t test
β; Chi Square test

Table 3. Endometriosis grades after surgery.

		Patient n (%)
Diagnosis	-	30 (60)
	+	20 (40)
Endometriosis grades	1	29 (58)
	2	5 (10)
	3	6 (12)
	4	10 (20)
Endometrioma	+	38 (76)
	-	12 (24)
Surgery	Laparotomy	36 (72)
	Laparoscopy	14 (28)

+: Yes; -: No; n: number.

Gal-3 has been reported to be highly expressed in ectopic, ectopic endometrium of patients with endometriosis compared to the control group (18). Especially, Gal-3 has been implicated in the development of neoplasms, including gynecological cancers (29).

The literature review revealed that Gal-1 and Gal-3 are overexpressed in the eutopic endometrium of women with endometriosis (5, 14), and Gal-3 in endometrial cells (30, 31). Based on these findings, we thought that Gal-3 expression might be abnormal in endometriosis patients.

In this pilot study, we revealed the potential for Gal-3 to be used as a biomarker in clinical practice in endometriosis. This study indicated that Gal-3 levels in the serum of women with endometriosis are remarkably increased compared with the control group. In addition, we showed that Gal-3 levels were increased in the 3 and 4 stages of endometriosis compared to the control group ($p < 0.05$). In previous studies, it was stated that Gal-3 levels increase in endometriosis. Noel et al. showed that Gal-3 expressions were increased in the proliferative or secretory phases of endometriosis compared to the eutopic

endometrium. Their study suggests that Gal-3 can play role in the development of endometriosis (18). In another study, Caserta et al. found that the expression of Gal-3 in peritoneal fluid of women with endometriosis increased significantly compared to controls (5). Mattos et al. investigated the effects of Gal-3 on the development of endometriotic lesions. They demonstrated that Gal-3 importantly contributes to endometriotic lesions development. (21). The same group showed that Gal-3 expression increased in experimental peritoneal endometriotic lesions (32). Our results support these data, Gal-3 expression levels were significantly overexpressed in stage 3-4 in endometriosis.

Even though the study includes a restricted sample and reported initial results, future studies are required to define the complete role of Gal-3 levels, and to determine biomarkers of diagnosis in endometriosis.

CONCLUSION

According to the results of this study, Gal-3 has roles in the development or progression of endometriosis, and gives us information about the severity of the disease in the advanced stages of endometriosis. However, it is clear that detailed further studies and more examples are needed to confirm these results.

Ethics Committee Approval: The study approval was obtained from the Ethics Committee of Cumhuriyet University Faculty of Medicine (No:2019-10/06).

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Conflicts of Interest: The authors declare no conflict of interest.

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ORIGINAL ARTICLE

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Significance of USP7 in Predicting Prognosis of Mammary Ductal Adenocarcinoma in the Turkish Population

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ABSTRACT

Objective: Breast cancer has the highest incidence and mortality rate among women worldwide. This study aims to analyze ubiquitin specific protease 7 (USP7) levels in mammary ductal adenocarcinoma patients in the Turkish population, to identify whether there is a relationship between the levels of USP7 protein and bad prognosis, which is indicated by enlarged tumor size, younger age, and receiving neo-adjuvant treatment.

Materials and Methods: In our study, we analyzed USP7 levels by immunohistochemistry (IHC) staining in 38 mammary adenocarcinoma cases in the Turkish population. Corelation analyses were performed to evaluate the distribution of the patients by their age, tumor size, Ki-67 levels, and the status of neo-adjuvant treatment by their USP7 levels. The IHC data concluded that the average age and tumor size are reversely proportional to the USP7 levels, insignificantly. The differences between the levels of USP7 and Ki-67 measurements were found to be statistically insignificant between the groups.

Results: Women who received the neo-adjuvant therapy prior to the operation presented much lower amount of USP7 levels when compared to their non-treated counterparts.

Conclusions: These findings highlighted that mammary ductal adenocarcinoma patients in the Turkish population present varying USP7 protein levels. Even though the levels of UPS7 are partially insignificant, the presence of USP7 protein might be a prognostic indicator in breast cancer patients in the Turkish population with a larger study set.

Keywords: Ubiquitination, USP7, mammary ductal adenocarcinoma, the Turkish population

INTRODUCTION

Breast cancer, one of the most lethal diseases among women, has the highest incidence and mortality rate that accounts for 25% of all cancer cases (1,2). Women diagnosed with breast cancer receive advantages from the latest therapeutical approaches; however, the incidents and cancer deaths continue to be a significant problem around the globe (3,4). Various parameters play a role in tumorigenesis, including family inheritance, lifestyle, age, menopause, and hormonal therapy. Mammary ductal adenocarcinomas differ widely due to the various subtypes based on the presence of Her2 receptors, treatment with a neo-adjuvant therapy, metastatic ability, and the expression of steroid hormone receptors such as progesterone and estrogen (5). Due to its complex structure at the molecular level, the treatment strategies against these carcinomas stay as troublesome. Although treatment methods vary,

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finding the best option stands as challenging, and therefore alternative targets are in urge.

One of the mechanisms that steer tumorigenesis is the dysfunction of protein stability. Ubiquitin–proteasome pathway is one of the major pathways that maintain this stabilization process. Not only just degrading but also targeting other proteins that signal various cellular processes, ubiquitins are essential for the development of cancer. We know that enormous amounts of proteins do play a role in these ubiquitin pathways. Ubiquitination is a post-translational process in which enzymes are involved and an attachment of a ubiquitin-protein to another protein. This is usually done by binding of last amino acid of ubiquitin to a lysine residue on the proteins. Ubiquitin–specific protease 7 (USP7), a member of a family of proteases, is involved in removing these ubiquitins and preventing protein degradation. For a USP7 to be activated, C-terminal ubiquitin-like domains are required to be folded back into the catalytic domain, allowing the active site to be adapted to a catalytically suitable state by the C-terminal peptide. Several substrates are targeted by USP7 that play a role in a diversity of cellular events, including cell cycle, chromatin remodeling, and DNA repair. Upon its abnormal activation, USP7 can trigger or suppress tumorigenesis proposing this particular protein a double-edged sword in tumorigenesis. Up to now, many therapeutics have been used to target USP7 in the treatment of cancer, make this molecule a promising candidate for cancer therapy.

MATERIALS AND METHODS

Patients and Tissue Samples

Thirty-eight mammary adenocarcinoma patients, who had been regularly followed up between 2018 and 2020 at the Department of General Surgery in Okan University Hospital, and whose slides and paraffin blocks were extracted from the archives of the Department of Pathology, were evaluated in the study. We collected all the samples by the approved ethical standards of the responsible committee of Yeditepe University Hospital. During the follow-up, we obtained the patient information about their age, tumor size, and receiving any neo-adjuvant therapy.

Immunohistochemistry

Immunohistochemical (IHC) staining was achieved to investigate the expression of USP7 in breast cancer tissues. Collectively tissues went under a series of processes, including fixation in 10% buffered formalin, embedding in paraffin, and further slicing in 4-µm thickness. Deparaffinization by incubating at 70°C for 15 minutes was followed by the dehydration step with xylene three times. Later, sections were rehydrated gradually with diluted alcohol solution ranging from 100% to 70%. Samples were then washed with phosphate-buffered saline (PBS) two times for 5 minutes and permeabilized in a solution containing tri-sodium citrate dehydrate and triton X-100 for 8 minutes at +4°C. Upon washing with PBS, samples were treated with antigen unmasking solution (pH: 6) in the microwave for 1 minute. Samples were washed sequentially with a pre-cooled PBS and room temperature (RT), respectively. Endogenous peroxidase activity was inhibited by incubating the sections in 0.3% hydrogen peroxide (H_2O_2) for 30 minutes at RT. Upon washing, the samples were treated with goat sera and incubated in a humidified chamber for 30 minutes. Primary antibody USP7 (ab108931, Abcam, USA) was applied at 4°C overnight. Subsequently, diaminobenzidine (DAB) reaction and peroxidase (HRP) detection were conducted with SignalStain® DAB Substrate Kit (8059, Cell Signaling Technologies, USA) according to the manufacturer's instructions. Lastly, all sections were counterstained with Gill's hematoxylin solution (1051740500, Sigma Aldrich, USA), dehydrated, air-dried, and mounted.

Statistical Analysis

In the beginning, the conditions for ensuring the normality assumptions of the age variable, tumor size, and Ki-67 levels were examined by looking following parameters; Skewness-Kurtosis values, Shapiro-Wilks significance value due to the sample size being 50 or below, and Q-Q plot chart. After the normality examination, the age variable was analyzed with a one-way ANOVA test, which is one of the parametric tests. Tumor size and Ki-67 level variables were examined with a non-parametric Kruskal-Wallis test. Lastly, the Chi-square test was applied to data that belonged to the status of neo-adjuvant treatment because of comparing two categorical groups. The p-value was taken as < 0.05 in all analyses.

RESULTS

Evaluation of Immunohistochemical Staining

Figure 1 (Figure1, A-C) demonstrates that based on the staining levels of the nuclei. The power of staining of each sample was imaged and depicted as follows. The level of staining for sample 1 showed the lowest staining, while the second and third samples expressed the medium, the most levels of staining, respectively.

Immunohistochemical Findings Related to Age, Tumor Size, Ki-67 levels, and Neo-adjuvant Treatment

Based on the intensity of USP7 staining, the distribution of people and their corresponding parameters of interest were depicted in Table 1 accordingly.

Skewness-Kurtosis values of the age variable met the criteria of normal distribution (6). Considering the significance value of the Shapiro Wilk result, it was seen that the age variable was normally distributed (p=0.497). The distribution of the age variable within the group was controlled by looking at the Q-Q Plot chart. It is seen that the series has a linear distribution, provides a normal distribution due to the absence of outliers. After normality examination, one-way ANOVA showed that the mean age decreases as the USP7 level increases although not significantly.

For the tumor size and Ki-67 levels, Skewness-Kurtosis, Shapiro-Wilk statistical value was invalid for normal distribution. Q-Q

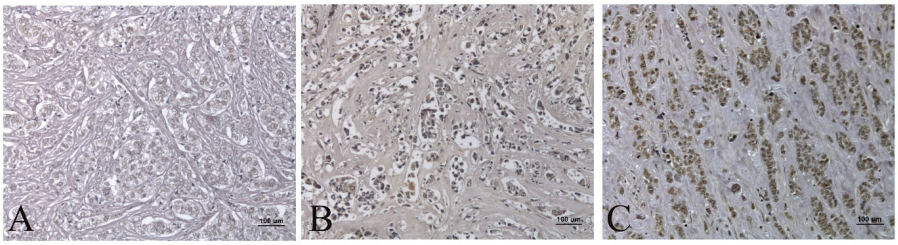

Figure 1. Images were taken with a light microscope with various staining levels of the nuclei (20x). A: Weak nuclei staining by USP7, score 1, B: Medium value nuclei staining by USP7, score 2, and C: Strong nuclei staining by USP7, score 3.

plot of these variables had non-linearity. Kruskal-Wallis results showed that the patients who possess medium, high levels of UPS7 levels had tumor sizes bigger than 20 mm. On the other hand, low levels of USP7 were observed among the ones with tumor sizes less than 20 mm.

As an important proliferation marker, only one out of 38 patients who had the Ki-67 measurement less than 10% was found to express low levels of USP7 whereas 7 of them had medium and high levels of USP7. Eight patients who had the Ki-67 measurement more than 10% had the lowest level of USP7, however, 22 of them had the medium and high levels of USP7.

Women who received the neo-adjuvant therapy before their operations were also subjected to the quantifications of USP7 levels. Accordingly, it seems that neo-adjuvant therapy overall has a downregulating effect on the USP7 levels. Out of 38 patients who had received the therapy, 2 patients expressed low USP7 levels while 4 of the total numbers of patients expressed USP7 levels at medium/high levels. Patients who did not receive any treatment expressed higher amounts of USP7 levels.

DISCUSSION

Discovered by Everett et al., the herpes virus-associated ubiquitin-specific protease (HAUSP), also known as USP7, was characterized in the late 1990s (7). This enzyme is involved in many cellular disorders, including cancers, neurological and metabolic syndromes, and immune dysfunctions. P53 is one of the substrates bound by this enzyme, and the reason for it to have a tumor suppressor role causes a decrease in the growth of tumor cells (8). However, further studies showed that mouse double minute 2 homolog (MDM2) was regulated by HAUSP-mediated deubiquitination, caused degradation of p53, reactivation of survival of the tumor cells (9). A recent study indicated the effect of USP7 on promoting the chemoresistance of triple-negative breast cancers (10), hence USP7 inhibitors could be potent agents in inhibiting several cancers (11). Therefore, the double-sword effect of USP7 stands as an open field to study in deep.

In our study, we have examined a total of 38 patients in the Turkish population based on their USP7 protein levels in histological specimens. An extensive analysis of the tumor size and its relationship with the levels of USP7 was performed. Although the size of the tumor might correlate inversely with

Table 1. Distribution and USP7 staining of all patients in accordance with the parameters including age, tumor size, Ki-67 levels, and receiving neo-adjuvant therapy.

Parameters	Limits	USP7 Levels			Total number of cases (n)
		Low (1)	Medium (2)	High (3)	
Age	< 40	0	3	2	38
	≥ 40	11	12	10	
Tumor size	< 20 mm	5	4	2	38
	≥ 20 mm	7	12	8	
KI-67%	≤ 10%	1	4	3	38
	> 10%	8	11	11	
NEO-ADJ treatment	-	8	13	11	38
	+	2	1	3	

the levels of USP7, no significant relationship was detected. The overexpression of USP7 levels in patients with epithelial ovarian cancer holds a bad prognosis and therefore, a high prognostic value in prediction (12). In addition, enhancer of zeste homolog 2 (EZH2) upregulation along with the high expression of USP7 was found to be related to bad prognosis in laryngeal squamous cell carcinoma (13).

One of the first parameters that we have taken into consideration is the age of the patients. According to our data, the average age is found to decrease while the USP7 protein level increases. These findings correlate with others as they explain how women at younger ages tend to present with breast cancer at an advanced stage than older ones, ultimately elucidating the worse outcome (14).

Ki-67 is one of the most valuable biomarkers in predicting tumor progression. A previous study indicates that Ki-67 might be a prognostic marker in breast cancer patients concerning the process and recurrence. Although we had the highest mean value of the Ki-67 measurement at a medium level, the lowest mean value at a low level, it was seen that the difference between the groups was not statistically significant (p=0.324).

In our study, we wanted to inspect whether USP7 levels could have a prognostic relevance of USP7 in breast cancer patients treated with neo-adjuvant therapy. Our results indicate that upon receiving neo-adjuvant treatment, the USP7 levels of the patients were found to be much lesser than the ones who had not received the therapy prior to the surgery. A similar study done by Giovinazzi et al. confirmed that USP7 inhibition improved the outcome of chemotherapy response in larynx carcinoma patients who were resistant to taxane treatment (15). However, an opposing study done by Cartel et al. shows that an increased expression of USP7 might be related to the elevated chemoresistance in acute myeloid leukemia (AML) patient-derived xenograft (PDX) models treated with cytarabine (16). These two conflicting data, along with our findings, assure that USP7 might be a changing prognostic criterion depending on the tumor type. Even though the levels of UPS7 are partially insignificant, the presence of USP7 protein might be a prognostic indicator in breast cancer patients in the Turkish population with a larger study set.

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Ethical approval: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Yeditepe University of Medical Sciences under code KAEK:984.

Consent to Participate: Informed consent was obtained from all individual participants included in the study.

Consent to Publish: The authors affirm that human research participants provided informed consent for the publication of the images in Figure 1(A-C) and Table 1.

Availability of Data and Material: The authors affirm the data they share and its availability.

Author Contributions: Concept- E.A.; Design- E.A., D.B.; Data Collection or Processing- G.V., T.K., D.B.; Analysis or Interpretation- E.A., D.B., G.V., T.K.; Literature Search- E.A., D.B.; Writing- E.A., D.B.; Approval-D.B.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Large-Scale Proteomic Analysis of Patients with Type 2 Diabetes Mellitus and Atherosclerosis Using a Label-Free LC-MS/MS Approach

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ABSTRACT

Objective: Type 2 diabetes mellitus (T2D) is a metabolic disease whose molecular events have not yet been fully clarified. However, next-generation powerful molecular approaches such as mass spectrometry (MS)-based proteomics holds promise. In this study, we aimed to reveal the protein profile of serum samples obtained from patients with T2D and atherosclerotic cardiovascular disease using the high-resolution liquid chromatography (LC)-MS/MS system.

Materials and Methods: Immune depletion was performed for the top 12 abundant proteins in 10 µl serum samples taken from individuals. Then, tryptic peptides were obtained from total proteins by applying a digestion protocol. Accordingly, reduction, alkylation, and digestion with trypsin enzyme were carried out, respectively. Tryptic peptides were analyzed in an ultra-high-pressure LC-MS/MS system with a label-free proteomic approach. The raw data were processed using the software program.

Results: LC-MS/MS analyses revealed 120 proteins with significant expression changes. Some of these proteins were associated with inflammation, lipid transport, and oxidative stress, which are known to play an important role in T2D and its complications.

Conclusion: As a result, LC-MS/MS analyses highlighted the proteins that will provide predictions in the treatment and course of T2D. We believe that validation of these proteins with targeted proteomic approaches in a larger sample in further studies will contribute to the development of clinically usable panels.

Keywords: Diabetes, proteomics, serum, mass spectrometry, atherosclerosis

INTRODUCTION

Type 2 diabetes mellitus (T2D) is a complex disease in which the individual's proteome changes depending on metabolic and functional disturbances, and the pathogenesis is not fully understood. However, proteomic approaches are becoming increasingly important in advancing our understanding of the etiology and pathology of T2D, and its complications. Liquid chromatography–mass spectrometry (LC–MS)-based proteomic methods allow sensitive detection of altered expression of proteins in T2D-dependent disrupted signaling pathways (1,2). Various biological materials such as serum, plasma, and urine have been used in proteomic studies investigating the disease and its complications (3-5). Serum samples are of particular interest for biomarker discovery as they are easily obtainable biological materials and contain information on a large number of proteins in

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various tissues. However, highly abundant proteins such as albumin, immunoglobulins, haptoglobin, transferrin, and α1-antitrypsin in serum samples constitute more than 80% of the total protein content. These abundant proteins prevent the detection of low-abundance proteins in LC-MS/MS analysis. Various techniques and methods continue to be developed to overcome this limitation. Immunodepletion, enrichment, and electrophoretic or chromatographic fractionation are some of the methods used (6).

To date, various serum, and plasma proteins that may be associated with T2D, its complications have been identified using LC-MS/MS-based proteomic approaches. A recent study reported 62 proteins, mostly immune-related, with altered expression in serum samples from T2D patients (3). In another study, a significant correlation was found between the levels of circulating APOC2, C4A, CXCL7, DOCK2, LBP, and VTDB proteins, the degree of coronary artery stenosis in cardiovascular patients with T2D (7). According to the results of LC-MS/MS analysis combined with gel methods, fibrinolysis, complement-dependent immune responses, and inflammation-related proteins were prominent in plasma samples of T2D and atherosclerotic patients (4).

Despite the advanced methods and techniques, the findings obtained in serum samples are still insufficient to explain the molecular mechanism of the disease, and new studies are needed. In this context, in this study, we aimed to reveal the protein profile of serum samples obtained from patients with T2D and atherosclerotic cardiovascular disease using the label-free proteomics approach.

MATERIALS AND METHODS

Case Information

Twelve patients with T2D and atherosclerosis (2 female and 10 male), and 9 control subjects (2 female and 7 male) were enrolled in the study. The study was approved by the Ethics Committee of Medipol University (24.10.2018/585), conducted by the principles of the Helsinki Declaration, and informed consent was obtained from each patient. Following an overnight fast, peripheral blood samples of the participants were collected into EDTA-free tubes. The serum samples were obtained by centrifugation for 10 minutes at 3000 rpm. The mean age of the patients was 53 years (45 to 60 years) and there was no statistically significant difference with the control group (mean age 52.4±6.9 years).

Sample Preparation

Samples were prepared according to a label-free mass spectrometry-based protein quantification strategy. For the control group, three pools were generated from nine different healthy subjects before digestion. However, patient samples were prepared individually. According to the manufacturer's instructions, abundant proteins in serum were depleted with a Top 12 spin column. Briefly, 10µL of serum was added to each column and incubated at room temperature, with gentle end-over-end mixing. Then, the samples were centrifuged at 1000×g and the flow through were collected. The digestion protocol applied to obtain the tryptic peptide was similar to our previous study (8). An equal amount of protein for each sample was reduced with 20 mM DTT for 10 min at 95°C and then alkylated with 40 mM iodoacetamide for 20 min in the dark. Following the reduction and alkylation procedures, proteins were digested with trypsin at 37°C for 18 hours. Digested peptides were dried under a vacuum, and stored at -80°C.

LC-MS/MS Analysis and Data Processing

The peptide mixtures were separated by a reverse phase nano-flow liquid chromatography (Dionex UltiMate 3000, Thermo Fisher Scientific) and identified using high resolution mass spectrometry (Q-Exactive Plus Orbitrap, Thermo Fisher Scientific) equipped with an electrospray ionization source. The analyses were performed by selecting the previous nano-UPLC-ESI-MS/MS system parameters (8). For analysis, each sample was re-dissolved in 0.1% formic acid and then loaded onto a trap column. Following the trapping, peptide mixtures were eluted to a C18 analytical column and separated with a linear gradient of acetonitrile. The collected data range was 400–2000 m/z. MS raw files were processed with Proteome Discoverer (version 2.3; Thermo Fisher Scientific, Bremen, Germany). All data were searched against the human UniProt database (downloaded in Jan 2022) containing 203,711 protein sequences. For protein quantitation, the minora feature detector and precursor ions quantifier node was used in the workflow generated in the informatics program.

Statistical Analysis

To calculate the p value , a hypothesis test (ANOVA individual) based on the abundance of individual proteins and peptides was selected. Using cut-off criteria, a 1.5-fold increase, or a 0.65-fold decrease in expression was considered to be of biological importance. A p value <0.05 was considered to be significant. Benjamini–Hochberg correction for multiple testing was applied to the p values. STRING database (https://string-db.org/), Cytoscape, and CytoHubba plugin were used for protein interaction networks and classifications.

RESULTS

Using a label-free quantitative proteomics workflow, we quantified 1,860 unique peptides mapped to 336 proteins in 12 patients with type 2 diabetes and 9 control samples (peptide and protein FDR 1%). As seen in Figure 1, this approach allowed the detection of 120 differentially expressed proteins (DEPs) including 50 proteins up-regulated (≥ 1.5-fold) and 70 down-regulated (≤ 0.65-fold) between T2D and the control group (adj. p-value <0.05). These proteins are listed in Table 1 with more information. The protein interaction network constructed using 120 proteins showed that they are highly related proteins (Figure 2A). The top 10 hub genes were identified by CytoHubba. These hub proteins are given in Figure 2B. The 6 hub proteins were mostly associated with lipid transport. Apart from APOA2 (3.2-fold), lipid transporter proteins such as APOA1 (0.21-fold), APOA4 (0.08-fold), APOC3

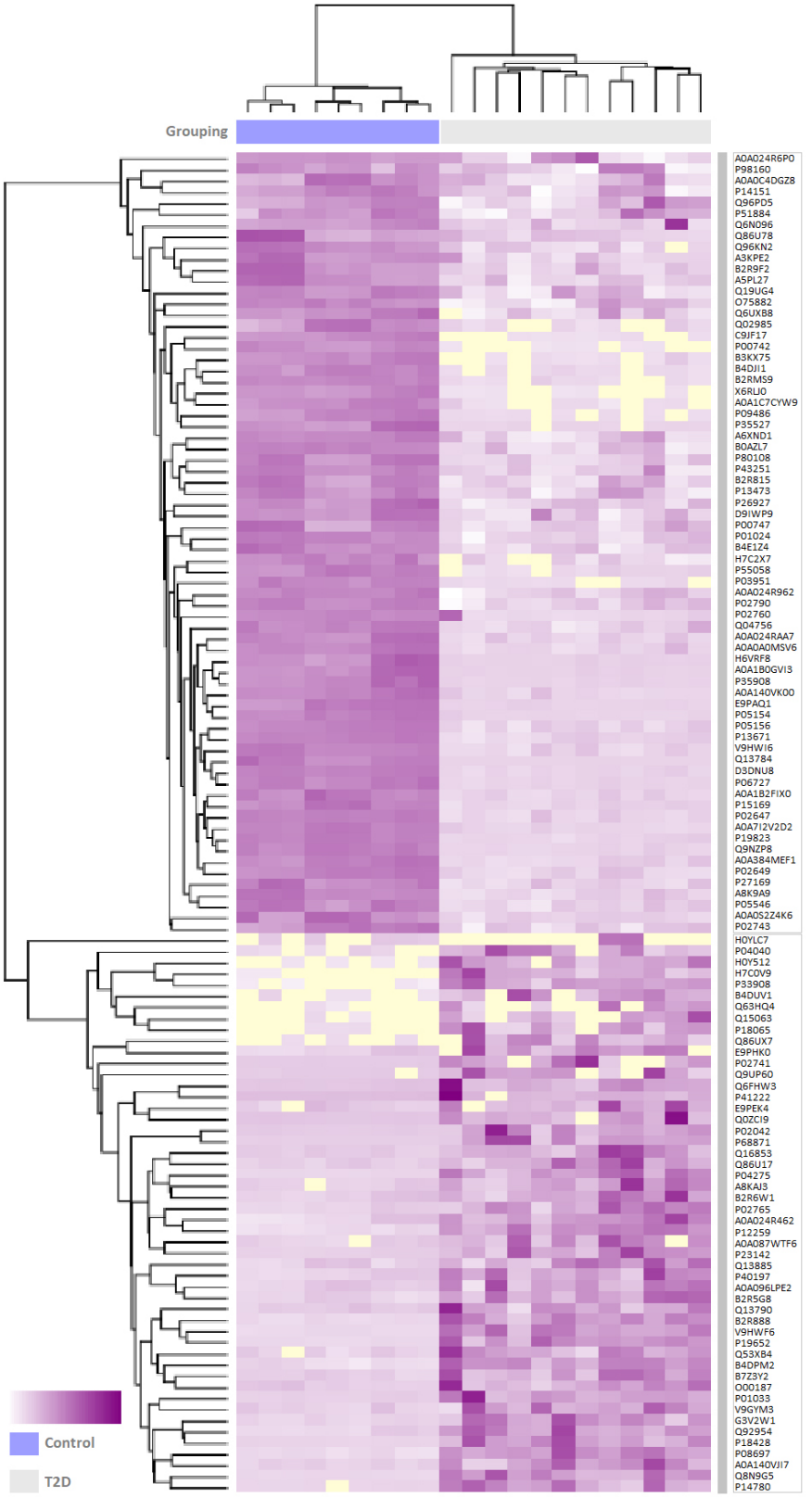

Figure 1. Hierarchical cluster analysis of the 120 DEPs. The proteins were clustered hierarchically by Manhattan correlation analysis. The greater the abundance of protein, the deeper the purple color. DEPs: Differentially expressed proteins.

(0.32-fold), APOD (0.03-fold), APOE (0.25-fold), APOH (0.37-fold), APOL1 (0.13-fold), and paraoxonase (PON)1 (0.27-fold) were significantly reduced (p<0.001) (Figure 3). Moreover, the level of C-reactive protein (CRP) (36.5-fold) as an inflammation marker was found to be higher compared to the control, whereas the level of adiponectin (0.21-fold), which is known to have an anti-inflammatory and antioxidant role, was found to be low (p<0.00001). However, we observed upregulation

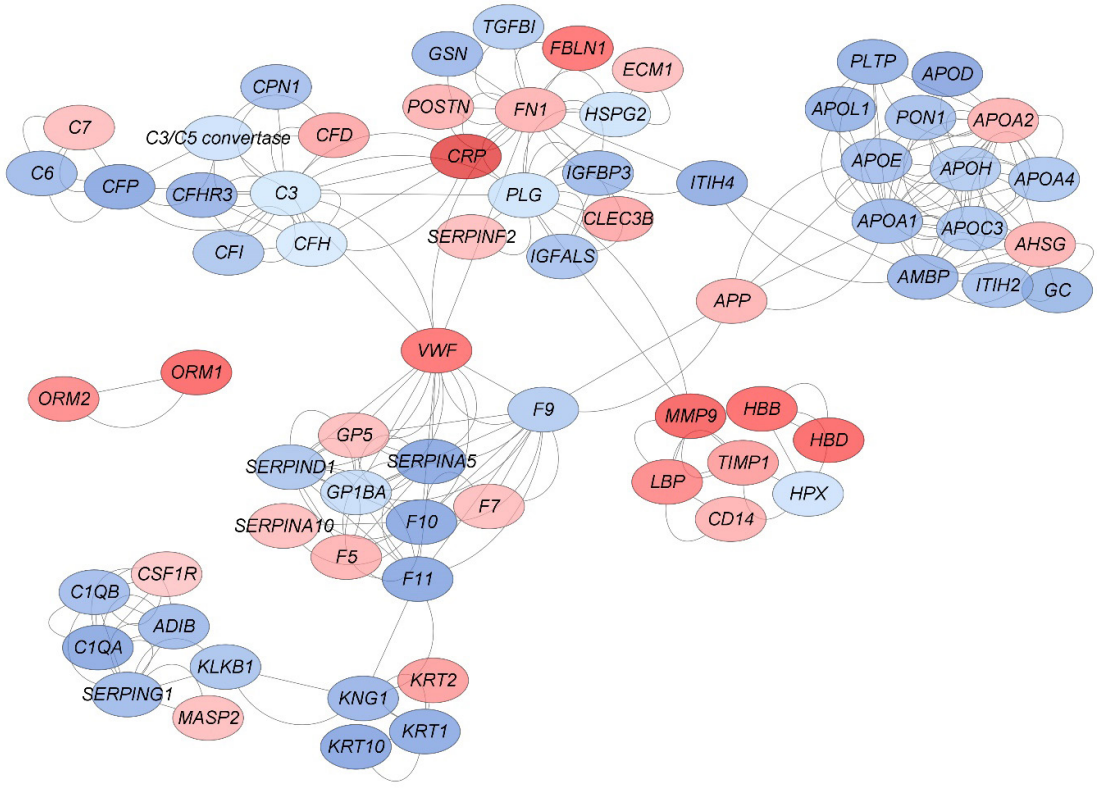

Figure 2A.

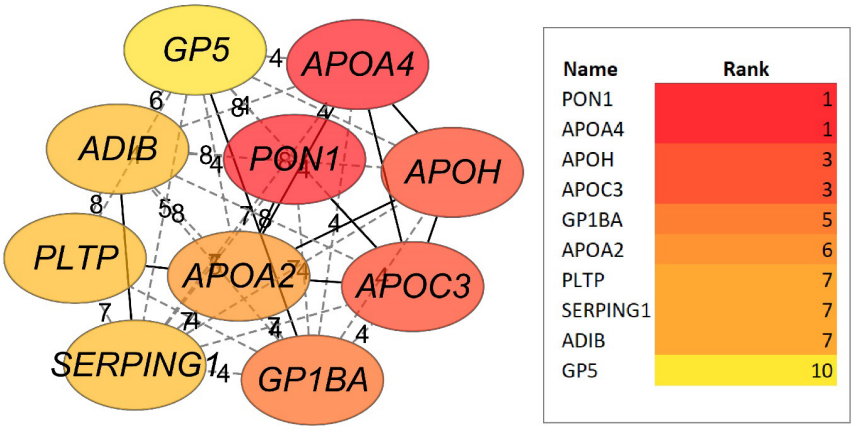

Figure 2B.

Figure 2. A. Protein interaction network of DEPs according to the STRING v11 database. B. The top 10 hub genes were identified by CytoHubba. The minimum required interaction score was selected with high confidence (0.900) and disconnected nodes were removed from the network. Average node degree: 2.1; avg. local clustering coefficient: 0.39; PPI enrichment p-value: < 1.0e-16. Blue indicates downregulated proteins, whereas red indicates upregulated proteins. The darker the color, the greater the difference in expression. DEPs: Differentially expressed proteins.

Figure 3A.

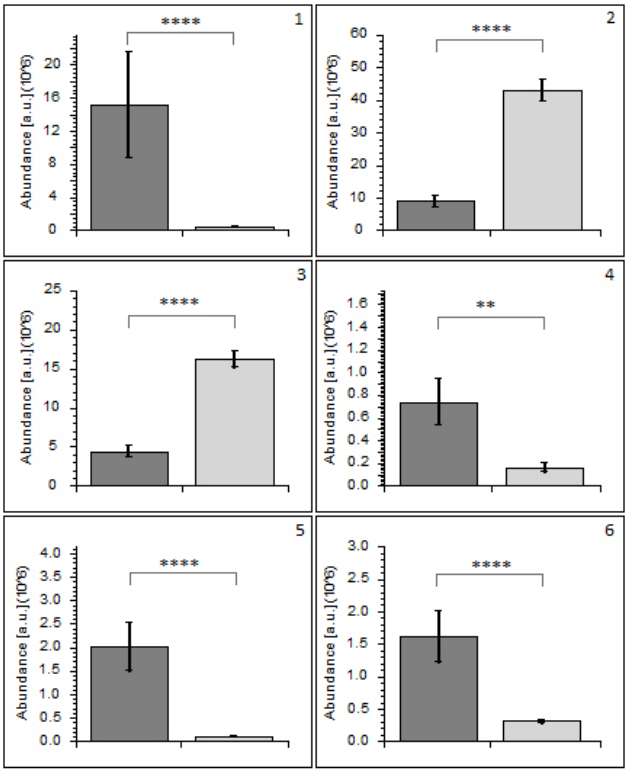

Figure 3B.

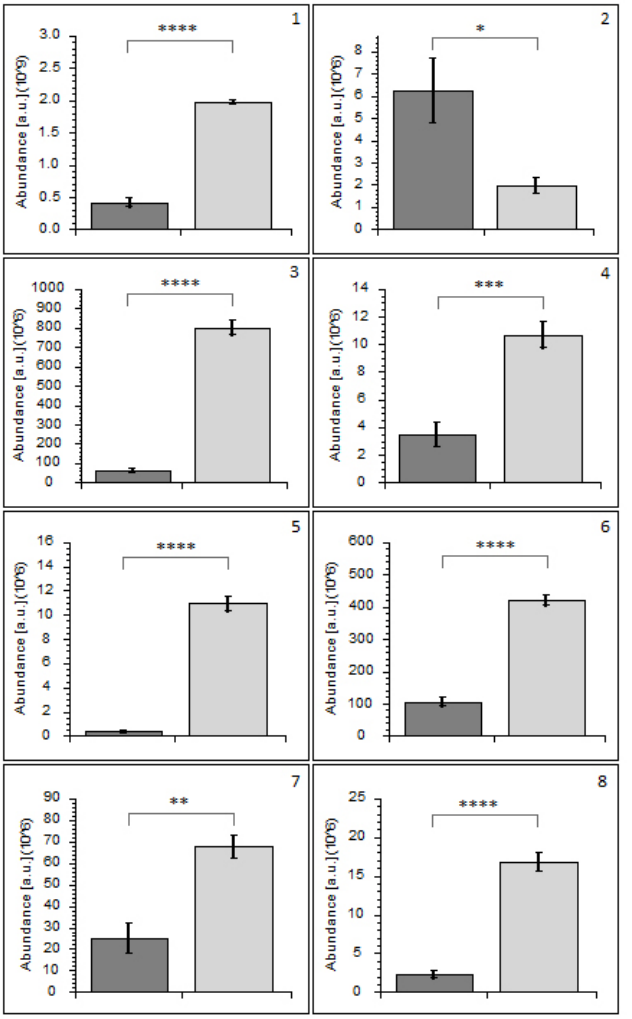

Figure 3. Bar chart reporting relative abundances of both six differentially expressed proteins (panel A: 1, CRP; 2, ADIP; 3, PON1; 4, CAT; 5, MMP9; 6, TIMP1) and eight proteins (panel B: 1, APOA1; 2, APOA2; 3, APOA4; 4, APOC3; 5, APOD, 6, APOE; 7, APOH; 8, APOL1), in patients and controls. All values are expressed as mean ± standard error of the mean (SEM). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Dark grey indicates the patient group, whereas light grey indicates the control group.

of catalase (4.5-fold), a known antioxidant protein (p=0.0026). Other significantly upregulated DEPs in our proteomic data were matrix metalloproteinase (MMP) 9 (17.9-fold) and tissue inhibitor of metalloproteinases (TIMP) 1 (5.17-fold) (p<0.00001) (Figure 3B). These two proteins belong to the protease inhibitor family and both of them have a vital role in the degradation of extracellular matrix and cell signaling.

We also performed gene ontology (GO) enrichment analysis to elucidate differentially expressed proteins' biological role. A total of 103 serum proteins were annotated, accounting for 85.8% of the DEPs. The classifications of DEPs in the proteomes of samples from T2D patients are given in Table 2. A significant portion of the DEPs were extracellular regions, vesicles, exosomes, blood microparticles, and collagen-containing extracellular matrix proteins. According to molecular function, the DEPs quantified are mainly involved in protein binding, ion binding, hydrolase activity, enzyme regulator activity, lipid binding, carbohydrate binding, and transporter activity. Interestingly, we found that the majority of proteins associated with lipid binding (F10, PON1, APOA1, APOC3, APOA4, SERPINA6, CPN1, AZGP1, SELL, APOH, APOD, APOE, APOL1, GC and PLTP), carbohydrate binding (ATRN, APCS, PGLYRP2, SERPIND1, SELL, APOH, KRT1, F11, APOE, KNG1, and SERPINA5) were down-regulated in T2D patients. In addition, the top 10 categories according to their significance are given in Table 2. Regarding the biological process, platelet degranulation, response to stress, complement activation, defense response, and regulated exocytosis were in the top 5 significantly enriched categories. Moreover, more than 50% of the proteins with increased expression were associated with response to stimulus and response to stress.

Table 1. List of significantly downregulated and upregulated proteins in patients with T2D and atherosclerotic cardiovascular disease relative to control, identified by LC-MS/MS analysis.

A-Downregulated proteins

Accession	Gene name	Description	FC	adj. p-value	MW [kDa]
A0A384MEF1	GSN	Actin-depolymerizing factor	0.19	8.7E-11	85.6
A0A024RAA7	ADIB	Adiponectin	0.21	3.3E-08	25.8
Q86U78	Serpin A8	Angiotensin 1-10	0.55	0.000	53.1
Q13784	APOA4	APOA4 protein (Fragment)	0.01	0.000	28.1
P02647	APOA1	Apolipoprotein A-I	0.21	0.000	30.8
P06727	APOA4	Apolipoprotein A-IV	0.08	0.000	45.3
A3KPE2	APOC3	Apolipoprotein C-III	0.32	0.001	10.8
C9JF17	APOD	Apolipoprotein D (Fragment)	0.03	0.000	24.1
P02649	APOE	Apolipoprotein E	0.25	0.000	36.1
D9IWP9	APOH	Apolipoprotein H (Fragment)	0.37	0.001	36.2
A0A1B2FIX0	APOL1	Apolipoprotein L1 (Fragment)	0.13	0.000	27.1
O75882	ATRN	Attractin	0.49	0.001	158.4
P98160	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein	0.58	0.027	468.5
Q96KN2	CNDP1	Beta-Ala-His dipeptidase	0.29	0.001	56.7
P43251	BTD	Biotinidase	0.55	0.000	61.1
B4E1Z4	n/a	C3/C5 convertase	0.57	0.000	140.9
P15169	CPN1	Carboxypeptidase N catalytic chain	0.25	0.000	52.3
B0AZL7	IGFALS	cDNA, FLJ79457, highly similar to Insulin-like growth factor-binding	0.28	0.000	66
B2R815	SERPINA4	cDNA, FLJ93695, member 4 (SERPINA4), mRNA	0.48	0.000	48.5
B2R9F2	SERPINA6	cDNA, FLJ94361member 6 (SERPINA6), mRNA	0.46	0.000	45.1
Q19UG4	F9	Christmas factor (Fragment)	0.36	0.001	20.7
P00742	F10	Coagulation factor X	0.04	0.000	54.7
P03951	F11	Coagulation factor XI	0.03	0.000	70.1
X6RLJ0	C1QA	Complement C1q subcomponent subunit A (Fragment)	0.05	0.000	23.3
A0A0A0MSV6	C1QB	Complement C1q subcomponent subunit B (Fragment)	0.23	0.000	24
Q9NZP8	C1RL	Complement C1r subcomponent-like protein	0.11	0.000	53.5
P01024	C3	Complement C3	0.64	0.000	187
P13671	C6	Complement component C6	0.21	0.000	104.7
Q02985	CFHR3	Complement factor H-related protein 3	0.15	0.000	37.3
P05156	CFI	Complement factor I	0.32	0.000	65.7
A5PL27	CP	CP protein	0.54	0.000	122.1
H6VRF8	KRT1	Cytokeratin-1	0.06	0.000	66

V9HWI6	HEL-S-51	Gc-globulin	0.18	0.000	52.9
A0A0C4DGZ8	GP1BA	Glycoprotein Ib (Platelet), alpha polypeptide	0.53	0.005	68.9
A0A024R962	hCG_40889	HCG40889, isoform CRA_b	0.65	0.000	139
P02790	HPX	Hemopexin	0.58	0.000	51.6
P05546	SERPIND1	Heparin cofactor 2	0.32	0.000	57
Q04756	HGFAC	Hepatocyte growth factor activator	0.32	0.000	70.6
P26927	MST1	Hepatocyte growth factor-like protein	0.43	0.000	80.3
B3KX75	CHL1	highly similar to Neural cell adhesion molecule L1-like	0.06	0.000	124.7
A6XND1	IGFBP3	Insulin-like growth factor-binding protein 3	0.21	0.000	29
P19823	ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	0.27	0.000	106.4
A8K9A9	KLKB1	Kallikrein B	0.29	0.000	71.3
A0A1B0GVI3	KRT10	Keratin, type I cytoskeletal 10	0.02	0.000	63.3
P35527	KRT9	Keratin, type I cytoskeletal 9	0.02	0.000	62
P35908	KRT2	Keratin, type II cytoskeletal 2 epidermal	0.04	0.000	65.4
D3DNU8	KNG1	Kininogen 1, isoform CRA_a	0.15	0.000	47.8
H7C2X7	LSG1	Large subunit GTPase 1 homolog (Fragment)	0.36	0.000	32
B4DJI1	n/a	L-lactate dehydrogenase	0.09	0.000	33.6
P14151	SELL	L-selectin	0.62	0.004	42.2
P51884	LUM	Lumican	0.56	0.008	38.4
P13473	LAMP2	Lysosome-associated membrane glycoprotein 2	0.51	0.000	44.9
Q96PD5	PGLYRP2	N-acetylmuramoyl-L-alanine amidase	0.58	0.034	62.2
Q6UXB8	PI16	Peptidase inhibitor 16	0.39	0.001	49.4
P80108	GPLD1	Phosphatidylinositol-glycan-specific phospholipase D	0.19	0.000	92.3
P55058	PLTP	Phospholipid transfer protein	0.13	0.000	54.7
B2RMS9	ITIH4	Plasma Kallikrein-sensitive glycoprotein	0.09	0.000	103.3
A0A7I2V2D2	SERPING1	Plasma protease C1 inhibitor	0.21	0.000	53.2
P05154	SERPINA5	Plasma serine protease inhibitor	0.04	0.000	45.6
P00747	PLG	Plasminogen	0.59	0.000	90.5
E9PAQ1	CFP	Properdin	0.04	0.000	45.1
P02760	AMBP	Protein AMBP	0.17	0.000	39
A0A1C7CYW9	TTLL8	Protein monoglycylase TTLL8 (Fragment)	0.23	0.000	94.5
A0A024R6P0	SERPINA3	Serpin peptidase inhibitor, member 3, isoform CRA_c	0.54	0.039	47.6
P02743	APCS	Serum amyloid P-component	0.48	0.000	25.4
P27169	PON1	Serum paraoxonase/arylesterase 1	0.27	0.000	39.7
P09486	SPARC	SPARC	0.03	0.000	34.6
A0A140VK00	n/a	Testicular tissue protein Li 227	0.12	0.000	34.2
A0A0S2Z4K6	TGFBI	Transforming growth factor-beta-induced protein ig-h3	0.38	0.000	57.3

B-Upregulated proteins					
Accession ID	**Gene name**	**Description**	**FC**	**adj. p-value**	**MW [kDa]**
P02741	CRP	C-reactive protein	36.5	0.000	25
P14780	MMP9	Matrix metalloproteinase-9	17.9	0.000	78.4
P02042	HBD	Hemoglobin subunit delta	14.1	0.002	16
V9HWF6	HEL-S-153w	Alpha-1-acid glycoprotein	13.3	6.1E-12	23.5
P18065	IGFBP2	Insulin-like growth factor-binding protein 2	13.2	0.011	34.8
P04275	VWF	von Willebrand factor	9.4	0.000	309.1
P68871	HBB	Hemoglobin subunit beta	9.1	0.001	16
B4DUV1	FBLN1	Fibulin-1	9.0	0.000	70.1
H0YLC7	FAH	Fumarylacetoacetase (Fragment)	8.6	0.000	18
P19652	ORM2	Alpha-1-acid glycoprotein 2	7.3	1.2E-10	23.6
A8KAJ3	EFEMP1	cDNA FLJ77823, highly similar to Homo sapiens EGF-containing fibulin-like extracellular matrix protein 1, transcript variant 3, mRNA	7.2	0.000	54.6
P18428	LBP	Lipopolysaccharide-binding protein	6.7	0.000	53.4
H0Y512	APMAP	Adipocyte plasma membrane-associated protein (Fragment)	5.8	1.3E-06	45.4
O43866	CD5L	CD5 antigen-like	5.6	0.008	38.1
P01033	TIMP1	Metalloproteinase inhibitor 1	5.2	0.000	23.2
P33908	MAN1A1	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	5.0	0.000	72.9
Q9UP60	SNC73	SNC73 protein	4.9	0.021	40.9
P23142	FBLN1	Fibulin-1	4.8	0.000	77.2
P04040	CAT	Catalase	4.5	0.006	59.7
P41222	PTGDS	Prostaglandin-H2 D-isomerase	4.4	0.026	21
Q92954	PRG4	Proteoglycan 4	4.0	0.000	151
E9PHK0	CLEC3B	Tetranectin	3.8	0.000	17.8
Q0ZCI9	n/a	Immunoglobulin heavy chain variable region (Fragment)	3.7	0.000	14.1
Q6FHW3	DF	Adipsin	3.5	1.6E-02	24.4
V9GYM3	APOA2	Apolipoprotein A-II	3.2	0.006	14.9
Q16853	AOC3	Membrane primary amine oxidase	3.1	0.000	84.6
Ferritin	FERMT3	Fermitin family homolog 3	3.1	0.010	75.9
P12259	F5	Coagulation factor V	3.1	0.000	251.5
Q8N9G5	n/a	cDNA FLJ37429 fis, clone BRAWH2001666	3.1	0.000	42.2
Q15063	POSTN	Periostin	3.0	0.037	93.3
P02765	AHSG	Alpha-2-HS-glycoprotein	2.9	0.000	39.3

B2R888	n/a	Monocyte differentiation antigen CD14	2.8	0.000	40
A0A087WTF6	NCAM1	Neural cell adhesion molecule 1	2.9	0.001	93.3
H7C0V9	APP	Amyloid-beta A4 protein (Fragment)	2.8	0.018	55.1
A0A024R462	FN1	Fibronectin	2.4	0.004	259
B7Z3Y2	PCYOX1	cDNA FLJ51879, highly similar to Prenylcysteine oxidase	2.3	0.001	48.3
B4DPM2	F7	cDNA FLJ55738, highly similar to Coagulation factor VII	2.3	0.000	43.6
A0A140VJI7	n/a	Testicular tissue protein Li 61	2.2	0.000	60.6
B2R5G8	n/a	Serum amyloid A protein	2.2	0.000	14.8
G3V2W1	SERPINA10	Protein Z-dependent protease inhibitor	2.2	0.000	55.1
B2R6W1	C7	cDNA, FLJ93143, highly similar to Homo sapiens complement component 7 (C7), mRNA	2.1	0.004	93.5
P40197	GP5	Platelet glycoprotein V	2.0	0.000	60.9
P08697	SERPINF2	Alpha-2-antiplasmin	2.0	2.4E-10	54.5
Q13790	APOF	Apolipoprotein F	1.9	0.029	35.4
E9PEK4	CSF1R	Macrophage colony-stimulating factor 1 receptor	1.9	0.003	74.2
Q53XB4	RAB1	Epididymis secretory sperm binding protein	1.8	0.015	16.8
Q86U17	SERPINA11	Serpin A11	1.8	0.001	47
A0A096LPE2	SAA2-SAA4	SAA2-SAA4 readthrough	1.7	0.026	23.3
Q13885	TUBB2A	Tubulin beta-2A chain	1.6	0.005	49.9
O00187	MASP2	Mannan-binding lectin serine protease 2	1.6	0.017	75.7

Proteins were listed along with their accession numbers (UniprotKB AC/ID). gene name. description. fold change. adj. p-values and molecular weight (Mw) in kilodaltons. The p-value adjusted using Benjamini-Hochberg correction for the false-discovery rate.

Finally, we showed the top 10 pathways in which DEPs play a role, with the protein interaction network generated in the STRING database based on the Reactome Pathways. Accordingly, platelet degranulation, hemostasis, platelet activation, signaling and aggregation, regulation of Insulin-like growth factor (IGF) transport and uptake by IGFBPs, post-translational protein phosphorylation, and complement cascade were among the remarkable pathways.

DISCUSSION

Type 2 diabetes is a metabolic disease that is initially silent and then causes micro- and macrovascular complications (9). Serum and plasma samples contain abundant proteins as well as numerous proteins of tissue origin. Therefore, proteomic analyses performed on serum samples are important to obtain information about proteins that play a role in the course of diabetes. To date, several biomarker candidates have been proposed for the early detection of diabetes using MS-based proteomic methods (1,2). Despite the results obtained, the molecular and cellular pathways associated with the disease and its complications are still not clear. In this study, we carried out label-free quantification and LC-MS/MS to identify the proteomic alterations in serum samples of atherosclerotic T2D patients. Differentially expressed proteins were generally associated with inflammation, oxidative stress, lipid transportation, and coagulation, and these changes were consistent with the literature. As is known, patients with T2D show a strong predisposition to atherosclerotic vascular diseases due to various factors. Increased oxidative stress and inflammation are the processes most blamed in this relationship (9,10). High levels of CRP, a well-known inflammatory marker, are also seen in the therapeutic monitoring of T2D and cardiovascular diseases (10,11). In addition, some studies have found an inverse correlation between blood CRP levels and PON1 and adiponectin levels (12-14). According to our MS analysis results, serum CRP levels increased 36.5 times in patients with atherosclerotic T2D compared to the control. Moreover, adiponectin and PON1 were found to be significantly lower, showing a negative correlation with CRP. Accordingly, the adiponectin level in the patients was significantly reduced (0.20-fold) compared to the control. It has been reported that adiponectin, which has anti-inflammatory, anti-atherogenic, and insulin-sensitizing effects, is decreased in T2D (12,15,16).

Table 2. Gene ontology (GO) Analysis of Differentially Expressed Proteins.

Term ID	Description	Gene Count	*p-Value*
Cellular Component			
GO:0005615	Extracellular space	103	4.09e-63
GO:0005576	Extracellular region	106	1.21e-56
GO:1903561	Extracellular vesicle	82	2.56e-49
GO:0070062	Extracellular exosome	81	1.34e-48
GO:0072562	Blood microparticle	32	1.17e-39
GO:0062023	Collagen-containing extracellular matrix	43	5.47e-39
GO:0031012	Extracellular matrix	46	3.86e-38
GO:0031982	Vesicle	87	2.69e-35
GO:0034774	Secretory granule lumen	26	2.23e-19
GO:0031093	Platelet alpha granule lumen	16	3.76e-18
Biological Process			
GO:0006950	Response to stress	69	8.44e-20
GO:0016192	Vesicle-mediated transport	45	1.04e-14
GO:0006952	Defense response	41	4.01e-16
GO:0045055	Regulated exocytosis	32	4.01e-16
GO:0051346	Negative regulation of hydrolase activity	26	3.69e-15
GO:0002576	Platelet degranulation	23	3.01e-22
GO:0010466	Negative regulation of peptidase activity	22	1.29e-15
GO:0006956	Complement activation	17	1.08e-19
GO:0006958	Complement activation, classical pathway	13	3.34e-15
GO:0072378	Blood coagulation, fibrin clot formation	12	3.36e-15
Molecular Function			
GO:0061134	Peptidase regulator activity	22	1.63e-16
GO:0030414	Peptidase inhibitor activity	20	7.72e-16
GO:0004866	Endopeptidase inhibitor activity	19	6.37e-15
GO:0004857	Enzyme inhibitor activity	23	6.89e-14
GO:0004867	Serine-type endopeptidase inhibitor activity	15	7.48e-14
GO:0030234	Enzyme regulator activity	29	1.09e-09
GO:0004252	Serine-type endopeptidase activity	13	6.90e-09
GO:0008201	Heparin-binding	13	2.23e-08
GO:0005539	Glycosaminoglycan binding	14	7.49e-08
GO:0070325	Lipoprotein particle receptor binding	7	2.27e-07

According to the order of p-adjust value, only the top 10 terms were displayed.

There is even growing evidence that high adiponectin levels in the blood are associated with a lower risk of type 2 diabetes and complications (14,17,18). PON1 also has antioxidant and anti-atherogenic activity like adiponectin. PON1, which is bound to serum HDL shows its antioxidant enzyme property by reducing the accumulation of lipid peroxide, which is responsible for the onset and progression of atherosclerosis (19). In our study, the level of PON1 was also found to be 0.27-fold lower in patients. It has been reported in a recent study that the decrease in PON1 activity and the lack of correlation with HDL and APOA1 may increase the potential risk for atherosclerosis-related diseases by causing HDL dysfunction (20). Some studies have suggested that PON1 activity may be dependent on HDL particle content (19,21). Fourteen different apolipoproteins that can be found in the structure of HDL and regulate cholesterol transport and metabolism have been identified (22). In our study, significant differences were found in serum levels of APOA1, APOA2, APOA4, APOC3, APOE, APOH, and APOL1 compared to the control. Of these, APOA1, APOA2, APOA4, and APOE are known as structural and functional apolipoproteins. The major structural proteins of the HDL-cholesterol complex are APOA1 and APOA2. It has been reported that APOA1, which is of interest due to its role in reverse cholesterol transport, shows a positive correlation with PON1 activity in serum samples of coronary artery patients (23).

Increasing evidence suggests that the micro and macro complications of diabetes may be related to the accumulation of free oxygen radicals and lipid peroxidation products (24). Malondialdehyde (MDA) is an important indicator of lipid peroxidation, and its increase has been reported in diabetic patients in many studies (25-30). However, the antioxidant status in patients with T2D is not as clear as the finding of increased oxidative products. Superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase are the primary antioxidant enzymes involved in the oxidant defense mechanism, and controversial reports have been reported regarding SOD and catalase enzyme levels. In some studies, no changes were detected in these enzymes, while a decrease or increase was reported in some studies (25-32). It has been reported in recent studies that the decrease in SOD activity indicates the presence of glycation and excessive lipid peroxidation in T2D (29,30). Other studies have reported that SOD activity is higher in serum and plasma samples of patients with T2D compared to controls. The authors interpreted this increase as an adaptive response to increased oxidative stress (27,31). According to the results of LC-MS/MS-based proteomic analysis performed by Andújar-Vera et al., a decrease in SOD1, SOD2, APOE, and APOM proteins, an increase in CAT and APOA4 proteins were detected in the calcified femoral arteries from 7 patients (32). In another study, catalase was significantly increased in the plasma of T2D patients with the absence and existence of nephropathy (29). These results suggest that a CAT increase in diabetic patients may be a compensatory mechanism against oxidative damage (25,29,32). In our study, the 4.5-fold increase in serum levels of catalase compared to the control group highlights the

supportive role of antioxidant enzymes. However, whether this alteration in catalase levels is related to the decrease in APOA1 and PON1 needs to be investigated further.

On the other hand, hyperglycemia in diabetic patients can increase the expression of MMP in macrophages and endothelial cells due to oxidative damage. MMPs are known as endopeptidases that target extracellular matrix proteins, some of which play an important role in remodeling venous tissue (33,34). Due to its role in diabetes, MMP9 is one of the most interesting MMP family members. In our study, the serum MMP9 level was found to be 17.9 times higher than the control. Various findings have shown that MMP9 level is significantly increased in macrophages and endothelial cells during hyperglycemia (35,36). The results obtained in these studies reveal that overexpression of MMP9 in particular is strongly associated with atherosclerotic plaque instability, which is an important risk factor for acute coronary syndrome (35-38). The activity of MMP9 is mostly controlled by TIMP1. Taken together, the effects of high glucose on MMP9 and TIMP1 expression may disrupt the MMP/TIMP balance. In addition, higher TIMP1 expression levels in endothelial cells and plasma are considered as an indicator of endothelial dysfunction (39,40). Inokubo et al. detected elevated MMP-9 and TIMP-1 in plasma samples of patients affected by acute coronary syndrome (41). Derosa et al. also reported that plasma levels of both MMP9 and TIMP1 were increased in a diabetic group (37). In agreement with their findings, our analysis revealed a significant increase in TIMP1 and MMP9 levels. Interestingly, the increase we observed in MMP9 expression was almost 4 times that of TIMP1. These findings may be an important sign in MMP/TIMP imbalance. In this context, both our study and the existing studies in the literature shed light on future studies by showing that MMP9 and TIMP1 may be new targets for the prevention of vascular complications in diabetic patients.

In addition, our LC-MS/MS analyses allowed the identification of several proteins, such as fibronectin (FBLN1), tetranectin (CLEC3B), periostin (POSTN), adipsin (DF), kininogen 1 (KNG1), lumican (LUM), SERPIND1, SERPINA6, SERPINF2, actin-depolymerizing factor proteoglycan (GSN), biotinidase (BTD) and fibulin (FBLN1), in agreement with the literature (39,42-46).

CONCLUSION

In summary, it has been emphasized in the literature that inflammation and oxidative stress may play an important role in T2D and its complications. The changes in CRP, adiponectin, PON1, MMP9, CAT, TIMP1, and apolipoproteins that we observed in our study support this view. Our study also showed that many proteins involved in interrelated mechanisms are differentially expressed in atherosclerotic patients with T2D. However, there is a need to confirm the expression changes detected in these proteins by different methods.

As a result, LC-MS/MS analyses highlighted the proteins that will provide predictions in the treatment and course of the disease. Quantification of these proteins with targeted proteomic approaches in a larger sample in further studies will contribute to the creation of clinically usable panels.

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Ethics Committee Approval: . The study was approved by the Ethics Committee of Medipol University (24.10.2018/585) and conducted by the principles of the Helsinki Declaration, and informed consent was obtained from each patient.

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The Comparative Molecular Typing of *Haemophilus Influenzae* Strains Isolated from the Adenoid and Tonsils in Patients Undergoing Adenotonsillectomy

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ABSTRACT

Objective: Microbiota in the upper respiratory tract begins to form immediately after birth. Aerobic and anaerobic flora will form when a healthy child reaches the age of five. Tonsillitis and adenoiditis are the most common upper respiratory tract infectious diseases in childhood and adulthood. Transmission of bacterial infections from tonsils to adenoid tissue (endogenous transmission) can be prevented by usage of suitable antibiotics. At the same time, continuous exogenous infections of each or both sites require adenotonsillectomy surgery. In this study, to understand the role of endogenous transmission of *Haemophilus influenzae* (*H. influenzae*) in adenotonsillectomy, we aimed to investigate the genetic relatedness of *H. influenzae* strains obtained from the tonsil and adenoid of the same patient.

Materials and Methods: Twenty eight patients were included the study for displaying the growth of 56 *H. influenzae* strains (28 isolates per site). We investigated the genetic relatedness of *H. influenzae* strains obtained from the tonsils and adenoids of the patients by using the pulsed-field gel electrophoresis (PFGE) method.

Results: Twenty one isolates were isolated from the tonsils and adenoids of unrelated patients. We excluded nine (32.2%) out of 28 patients' *H. influenzae* isolates from the study, that had identical strains depending on the ≥80% similarity Dice coefficient.

Conclusion: The probability of endogenous transmission between the two sites was very high, which means that some adenotonsillectomy surgery might be avoided and treated with antibiotics.

Keywords: Adenotonsillectomy, pulse field gel electrophoresis, *Haemophilus influenzae*

INTRODUCTION

Microbiota in the upper respiratory tract starts to evolve after birth immediately, aerobic and anaerobic flora form at the age of five. Tonsillitis and adenoiditis are the most common upper respiratory tract infectious diseases in childhood and adulthood. Frequent recurrence and chronicity of tonsil and adenoid infections could cause a high cost and loss of the workforce. In untreated cases, acute tonsillitis and adenoiditis complications may invade adjacent respiratory tract areas, and suppurative complications might develop (1-5). In our previously published study, we reported the distribution of microorganisms and their susceptibilities to different antibiotics in adenoids and tonsils in patients undergoing adenotonsillectomy (6). 21 of the isolates were taken from the tonsils and adenoids of unrelated patients and excluded from the study, whereas we included 28

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patients displaying growth of 56 *Haemophilus influenzae (H. influenzae)* strains (28 isolates per site). Transmission of bacteria between tonsils and adenoid tissue (endogenous transmission) refers to acute infection, and can be treated with suitable antibiotics with no need for adenotonsillectomy. The continuous infection of one or both sites with exogenous *H. influenzae* strains (exogenous transmission) means that these sites become infection foci, and indicates chronic infection and the need for adenotonsillectomy surgery. To understand the role of endogenous transmission of *H. influenzae* in adenotonsillectomy, we aimed to investigate the genetic relatedness of *H. influenzae* strains obtained from the tonsil and adenoid of the same patient and vice versa.

MATERIALS AND METHODS

Patients who were admitted to the Otorhinolaryngology Clinic of the Istanbul University, Istanbul Faculty of Medicine in Istanbul, Turkiye were examined. Patients who met the surgical criteria and had both tonsil and adenoid tissue removed at the same time were included in the study. A total of 100 patients (64 males and 36 females; mean age ± standard deviation, 7.625 ± 3.267 years) who had chronic adenoiditis/tonsillitis and did not receive any antibiotics for the previous two weeks were selected for adenotonsillectomy surgery and enrolled in the study. The ethical approval was obtained from the Istanbul University, Istanbul Faculty of Medicine Clinical Research Ethics Committee (08.03.2011/533).

Bacterial Isolation

The samples were examined in the bacteriology laboratory of Istanbul University, Istanbul Faculty of Medicine, Department of Medical Microbiology. All samples were homogenized with sterile saline (0.85% NaCl) solution and cultured in chocolate agar (Oxoid, England) for bacterial isolation. All cultures were incubated at 35°C in 5% CO_2 atmosphere for at least 24-48 hours. Gram-negative coccobacilli that required factors X and V (BD, USA) when tested on tryptic soy agar (BD, Germany) were identified as *H. influenzae,* stored for studies at -80 °C.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) is the accepted "Gold Standard" for bacterial typing (7). Genetic relatedness among tonsil and adenoid isolates of the same patient were investigated by PFGE (8, 9). Concisely, examples embedded in genomic DNA were digested with 20 U SmaI-digested (Takara, Japan) enzyme for two hours at 37°C. A lambda ladder PFGE marker (BioLabs, Ipswich, MA, USA) was used as a molecular measure ladder. An electrophoretic run was performed in 0.5X TBE buffer (90 mM tris, 90 mM boric acid, and 2 mM EDTA) under the following conditions: 6 V/cm^2 for 20 h, pulse times from 5.3 to 34.9s at 14°C, using CHEF DRIII (Bio-Rad Laboratories, Belgium). The gel was smudged with ethidium bromide (1µg/mL) for 30 min, imaged under UV light using a transilluminator, and imaged and stored as a TIFF file (Figure 1). Genetic imprints for separate strains were obtained from the GelCompar II system (version 6.0, Applied Maths, Sint-Martens-Latem, Belgium). They were approximated and analyzed using dendrograms of the band profiles constructed employing the mathematically averaged unweighted double cluster manner. The association of the isolates was determined by the membrane coefficient with a band position tolerance setting of 1-1.5%. If the Dice coefficient was ≥80%, the isolates were identified as the same type (clonal). Tenover criteria were used in the analysis of the bands (8, 9).

Statistical Analysis

Similarity analysis and clustering were calculated by the Dice coefficient method, assumed via an unweighted pairwise group mean relationship (UPGMA) with GelCompar II v.6.0. The isolates showing a similarity coefficient of ≥80% were considered to belong to the same cluster(7).

RESULTS

H. influenzae growth was detected in 86 (86%) patients out of 100 patients. However, only 28 (28%) patients were included in the study since growths in both tonsil and adenoid tissue would be compared.

Nine (32.2%) out of 28 patients' *H. influenzae* isolates had identical strains depending on the ≥80% similarity Dice coefficient. That is why it was thought that the probability of endogenous transmission between the two sites was very high, whereas both sites of the same patient were colonized or infected with 38 unidentical strains in 19 (67.8%) patients. More likely, their source of infection was exogenous (Table 1). Isolates with a Dice band-based resemblance coefficient value of ≥80.0% were thought to belong to the same cluster, which implied a two- to three-fragment dissimilarity in gels with an average of 12 bands. The nine identical strains were as follows: 11A-11T, 34A-34T, 78A-78T, 80A-80T, 90A-90T, 94A-94T, 100A-100T, 111A-111T, 114A-114T; PFGE agarose gel raw data results are given in supplementary data.

DISCUSSION

Adenoid and tonsil tissues are common sites of colonization for *H. influenzae*. Adenotonsillectomy, the surgical removal of both the tonsils and adenoids, is a commonly performed procedure to improve breathing in children with sleep disorders or recurrent infections.

In recent years, comparative molecular typing methods have been developed to differentiate between *H. influenzae* strains isolated from different anatomical sites or patient groups. These methods are based on the analysis of genetic material, such as DNA or RNA, to identify variations in the strains' genetic code.

Tonsillitis and adenoiditis are serious infections caused by different microorganisms. In another study of us, (6), we isolated 14 different pathogenic bacteria in the adenoid and tonsils of patients who underwent adenotonsillectomy and *H. influenzae* strains were the most dominant (31.8%) among

Table 1. PFGE analysis of *H. influenzae* strains between adenoid and tonsil tissue.

Tonsil Tissue	Adenoid Tissue	Genotype
10 T	10 A	Similar
11 T	11 A	Not Similar
20 T	20 A	Not Similar
34 T	34 A	Similar
37 T	37 A	Not Similar
39 T	39 A	Not Similar
41 T	41 A	Not Similar
44 T	44 A	Not Similar
45 T	45 A	Not Similar
56 T	56 A	Not Similar
65 T	65 A	Not Similar
73 T	73 A	Not Similar
78 T	78 A	Similar
80 T	80 A	Similar
81 T	81 A	Not Similar
82 T	82 A	Not Similar
90 T	90 A	Similar
91 T	91 A	Not Similar
94 T	94 A	Similar
95 T	95 A	Not Similar
97 T	97 A	Not Similar
98 T	98 A	Not Similar
100 T	100 A	Similar
102 T	102 A	Not Similar
110 T	110 A	Not Similar
111 T	111 A	Similar
114 T	114 A	Similar
118 T	118 A	Not Similar

them. This result prompted us to ask whether this bacterium transferred between two sites (endogenous source), or whether these sites had become a suitable environment for colonizing of *H. influenzae* strains a part from infection of sites (exogenous source). First outcome that was thought,, the treatment with a suitable antibiotic might work, and there would be no need for adenotonsillectomy. But in the second case, the use of antibiotics is a permanent choice, and adenotonsillectomy is evitable. For this purpose, we investigated the genetic proximity of 56 *H. influenzae* strains isolated from tonsils and adenoids belonging to 28 patients. We showed that nine (32.2%) patients' strains were identical (100% similarity), and 19 (67.8%) were not identical. The exogenous source of *H. influenzae* strains in this study was higher compared with the endogenous source. This result indicates that adenotonsillectomy was evitable in most examined patients.

In the study by Choi et al., microorganisms grown in tonsils and saliva samples isolated from pediatric patients who underwent tonsillectomy for the therapy of tonsil hyperplasia were compared and, the growing microorganisms were evaluated by the 16S rRNA sequence analysis method. 24.3% of growing *Haemophilus spp.* were isolated from tonsils and 10.4% from saliva (29 tonsil samples) (10). In our study, we evaluated both the surface and core parts of the tonsils together, and *H. influenzae* was detected in 86% of patients. When *H. influenzae* studies are examined, it has been observed that it also plays a significant role in tonsillar hyperplasia (10-12).

A study in 2019 reported on preschool children aged between three to five years with hypertrophy of the pharyngeal or palatine tonsils. It stated that during this period, behavioral and sleep quality deterioration may occur depending on the enlargement of the tonsils, physical development difficulties such as respiratory tract difficulties, apnea, and snoring development (13). Infections of the upper respiratory system might extend to include the middle ear and cause acute otitis media (AOM), and also extend to the lower respiratory system and cause more serious infections, such as pneumonia. Fuji et al (14) reported a study on AOM in 2021. They conducted their study on children aged 6 to 30 months. They studied samples from 565 healthy individuals and 130 acute otitis patients. *H. influenzae* was detected in 5.9% of healthy visiting and 27% of acute otitis visits. *H. influenzae* was sequestered in 43% of middle ear fluid (MEF) specimens. With the widespread use of the *H. influenzae* type b (Hib) vaccine, the number of cases has decreased. For example, in the USA, the introduction of the Hib vaccine in 1985 dramatically decreased the incidence of invasive Hib disease (14). The Hib vaccine was introduced in Turkiye recently compared with the USA. The Hib vaccine (under a year old) was launched in Turkiye in 2006 by the Turkish National Immunization Program (NIP). Amoxicillin is the antibiotic recommended as the first-line treatment forAOM in the USA and most European countries, including Turkiye. Today, most strains produce beta-lactamase, which inactivates this antibiotic. A beta-lactamase inhibitor such as amoxicillin/clavulanic acid is alternatively used in the treatment of AOM, tonsillitis, and pharyngitis. Chekrouni et al. (15) collected data for twelve years and determined that 4% of bacterial meningitis episodes were caused by *H. influenzae* (an annual incidence of 0.5 patients per 1,000,000). They identified the predisposing factors as otitis and/or sinusitis, with a rate of 49%. Community-acquired pneumonia might be the exogenous source of *H. influenzae*. But this requires more data, and our study was

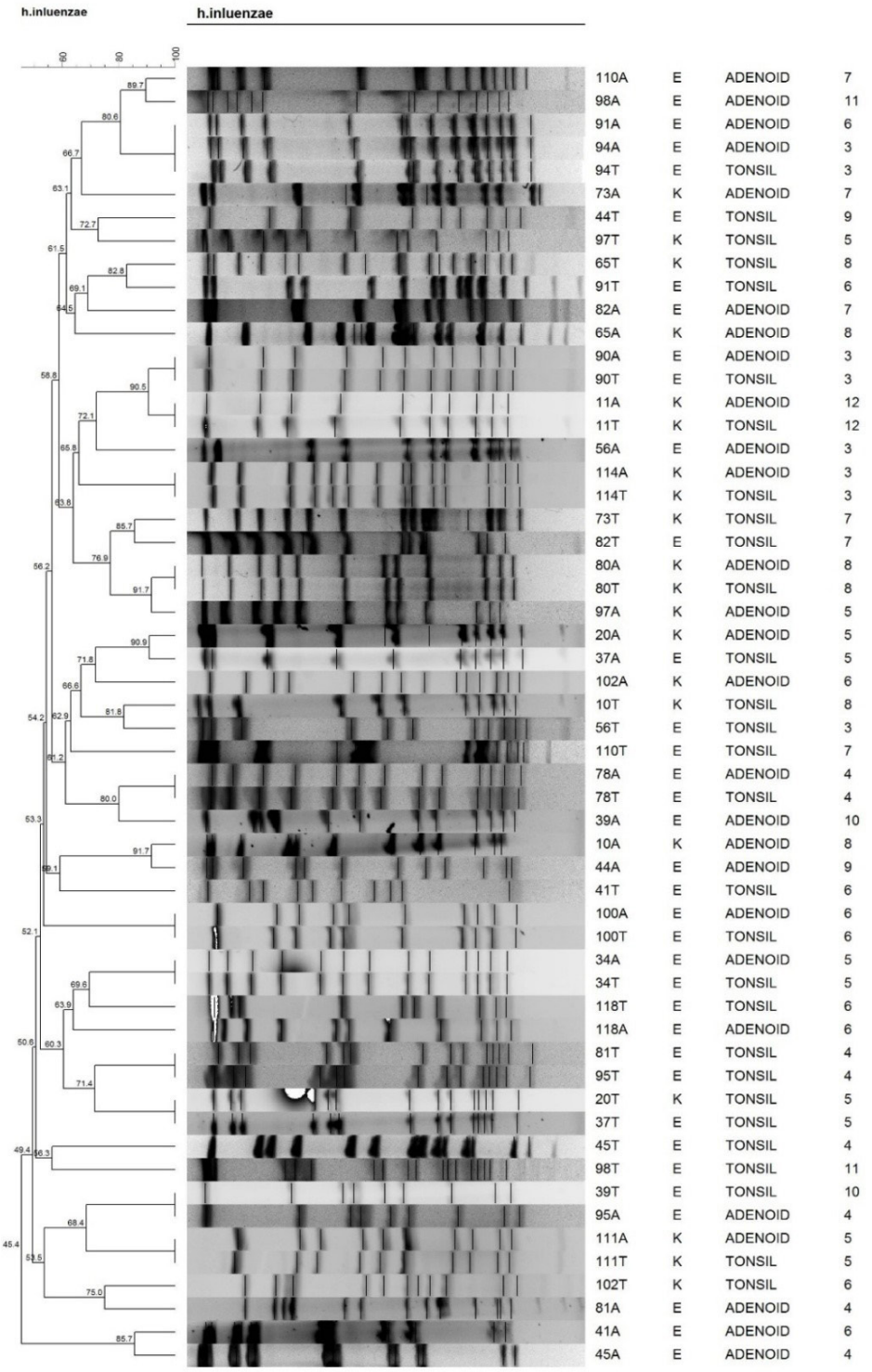

Figure 1. Dendrogram of *H. influenzae* by PFGE analysis by SmaI digestion. The membrane coefficients are shown above the dendrogram. Isolates with ≥80% on the dendrogram are considered highly genetically related.

not conducted to include *H. influenzae*-related pneumonia in hospitalized patients (15). When we investigated the studies in Turkiye (16), the microflora on the tonsillar surface, nucleus, and posterior surface have been compared. Only bacterial isolation techniques were used in this study, and 28% of *H. influenzae* strains were isolated (13). Unal et al. (14) compared the superficial and deep tonsillar flora, and the *H. influenzae* strain was found at a rate of 12% (17).

Our study compared *H. influenzae* strains isolated from adenoid tissue and tonsils, typified by conventional methods. However, when we discussed the previous studies, we could not find any comparative study of these two tissues. Hadi et al. (18) compared tonsil and adenoid tissue as superficial tissue cultures. On the other hand, we compared direct tissue cultures from both regions. A similar result was obtained in our study, which detected *H. influenzae* as a reproduction (15).

CONCLUSION

The endogenous transmission in this study was 32.2%, which means that some adenotonsillectomy surgery could be avoided and treated with antibiotics. Tonsillitis and adenoiditis are most frequent childhood diseases. Some children in this age group experience various physical and cognitive problems. We aimed to shed light on the relationship between these two close tissues. Also, we wanted to be a pioneer in studies to be carried out on this subject.

Ethics Committee Approval: The ethical approval was obtained from the Istanbul University, Istanbul Faculty of Medicine Clinical Research Ethics Committee (08.03.2011/533).

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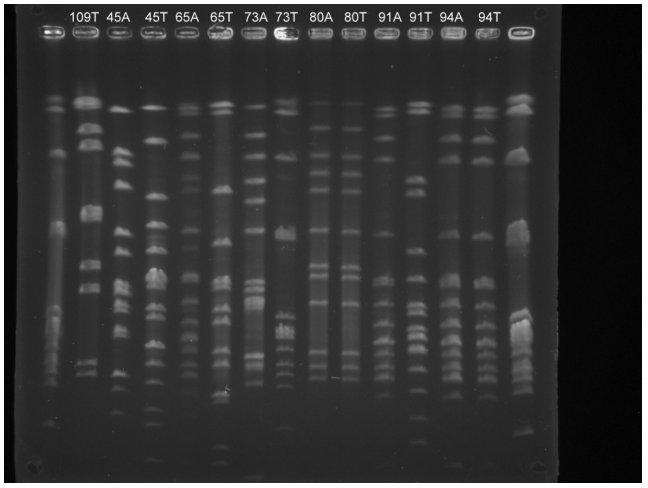
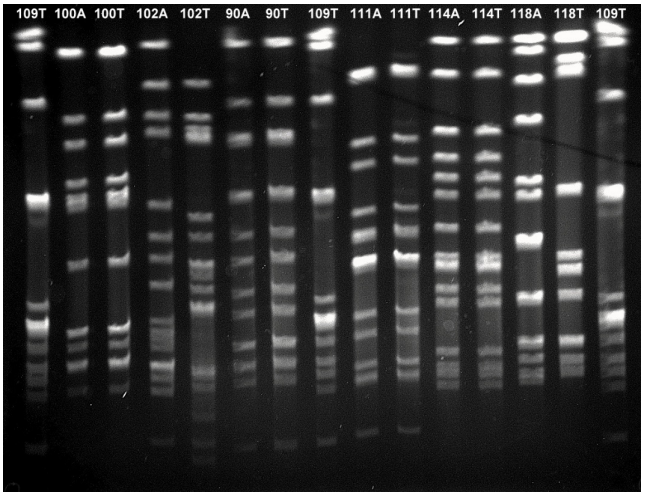
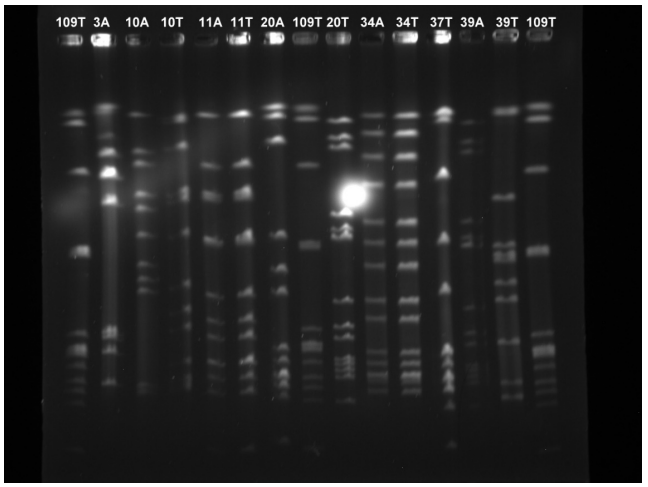
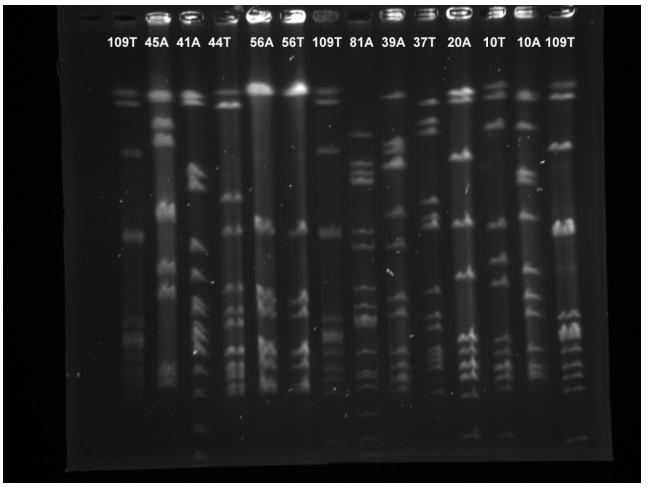
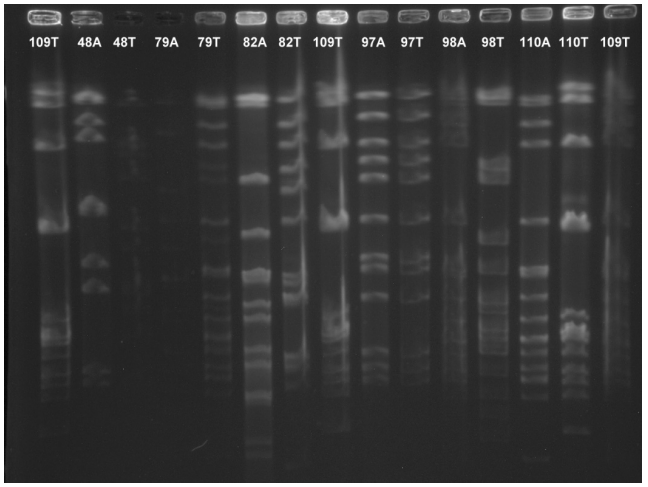
Supplementary Data: PFGE agarose gel raw data results.

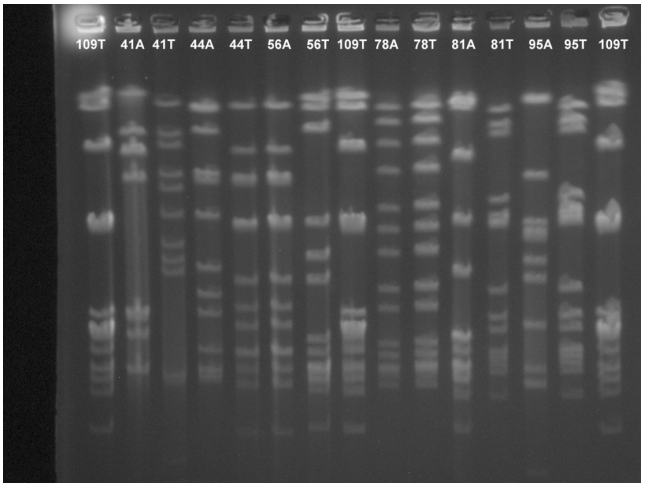












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A Bioinformatics Analysis of circRNA/miRNA/mRNA Interactions in Acute Myeloid Leukemia

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ABSTRACT

Objective: Acute myeloid leukemia (AML) is a lethal type of cancer associated with dysregulation of progenitor hematopoietic stem cell behavior and its incidence is, unfortunately, increasing. Although there are various applications in treatment, since most of them are insufficient in early diagnosis, treatment and new prognostic biomarkers should be investigated.

Materials and Methods: In this study, three Gene Expression Omnibus (GEO) datasets Genomic Spatial Event (GSE); GSE94591, GSE116617, and GSE163386) were used to investigate dysregulated expressions of circular RNAs (circRNAs), and the GSE142699 and GSE142698 datasets were analyzed to detect dysregulated expressions of microRNAs (miRNAs) and mRNAs, respectively. Filtering was applied with p value <0.05, log2FC≥0.5 (circRNA), and log2FC≥1 (miRNA and mRNA) from the raw data analyzed using the limma R package (v.3.46.0). We investigated circRNA-miRNA-mRNA interactions using special tools including CSCDv2.0, circBank, miRTarBase, miRDB, multiMiR, miRWalk, DIANA-microT, TarBase, miRanda, and TargetScan. The pathway analysis was performed using KEGG and GO programs. The STRING database and Cytoscape tool were used to construct and view protein interaction. Hub gene analysis was constructed using the MCODE tool. We have utilized the GEPIA tool to determine the Overall Survival of the hub genes.

Results: In our study, 4 circRNAs, 3 miRNAs, and 6 genes that may be closely related to AML were detected.

Conclusion: According to our bioinformatics analysis results, hsa_circ_0012152/miR-199a-5p/*HOXA9* axis could be more important in AML. Therefore, *in vitro and in vivo* investigations are recommended.

Keywords: Acute myeloid leukemia, circular RNA, bioinformatics

INTRODUCTION

Acute myeloid leukemia (AML) is a hematological cancer that affects mostly adults and has a complicated classification and prognosis. There are only a few target therapeutic molecules for AML, and the necessary success in treatment has yet to be reached (1-3) . There is a need for new diagnostic and therapeutic target molecules for AML, which has become more complex due to the wide variety of genetic and epigenetic changes that occur (4). It has been reported that more than 20,000 people are diagnosed with AML every year in the United States and approximately 11,000 people die due to AML (5). Non-coding RNAs regulate gene expression during the post-transcriptional process (3, 6, 7). Long non-coding circular RNAs (circRNAs), which have a wide variety of functions in the cell, control gene expression by sponging miRNAs. circRNAs have a more stable structure than linear RNAs and are among the important research topics of the last 5 years. Dysregulation of the expression of circRNAs has been reported in a wide variety of cancer types, including lung cancer, prostate cancer, gastric cancer, and breast cancer, and also AML (8,

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9). It has been revealed that circRNAs play crucial roles in the pathogenesis of AML through various axes such as circRNA-DLEU2/miRNA-496/*PRKACB* and circ_0040823/miR-516b/*PTEN* (10, 11). In our study, a meta-analysis of circRNA, miRNA, and mRNA datasets in AML was performed with bioinformatics tools and circRNA-miRNA-mRNA axes that may be important in AML was determined.

MATERIALS AND METHODS

circRNA Expression Analysis of AML Datasets

Genomic spatial event (GSE); GSE94591 (4 healthy controls and 6 AML patients / bone marrow samples), GSE116617 (4 healthy controls and 4 AML patients/ bone marrow samples), and GSE163386 (4 healthy controls and 5 AML patients / bone marrow samples) gene expression omnibus (GEO) datasets were used to investigate dysregulated expressions of circRNAs. Filtering was applied with p value <0.05 and log2FC≥0.5 from the raw data analyzed using the limma R package (v.3.46.0). After identifying circRNAs with significant expression changes, their relationship with cancer and AML was investigated in the literature using "AML, hsa_circ_0012152" and "Acute myeloid leukemia, hsa_circ_0012152" parameters in PUBMED and other internet networks.

miRNA and mRNA Expression Analysis of AML Datasets

The GSE142698 and GSE142699 datasets were analyzed to detect dysregulated expressions of mRNAs and miRNAs, respectively. Filtering was applied with p value <0.05 and log2FC≥1 from the raw data analyzed using the limma R package (v.3.46.0).

Determination of circRNA and miRNA Interaction

CSCDv2.0 and circBank databases were used to identify miRNAs that could be sponged via selected circRNAs. The relationship between AML and these detected miRNAs was investigated in the literature using "AML, hsa_circ_0012152, miR-199a-5p" and " Acute myeloid leukemia, hsa_circ_0012152, miR-199a-5p" parameters in PUBMED and other internet networks.

Detection of miRNA-mRNA Relation

The GSE142699 dataset was analyzed to detect dysregulated mRNAs. Filtering was applied with p value <0.05 and log2FC≥1 from the raw data analyzed using the limma R package (v.3.46.0). miRTarBase, miRDB, multiMiR, miRWalk, DIANA-microT, TarBase, miRanda, and TargetScan databases were used for the prediction of selected miRNAs and possible target genes. By evaluating the literature data, genes that may be strongly associated with AML were identified.

Enrichment Analyses Via KEGG and GO Programs

The pathways analysis was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) programs. The search tool for the retrieval of interacting genes/proteins (STRING) database and Cytoscape tool were used to construct and view protein interaction. Hub gene analysis for selected miRNAs' possible target genes was investigated using the molecular complex detection (MCODE) tool with default parameters (9).

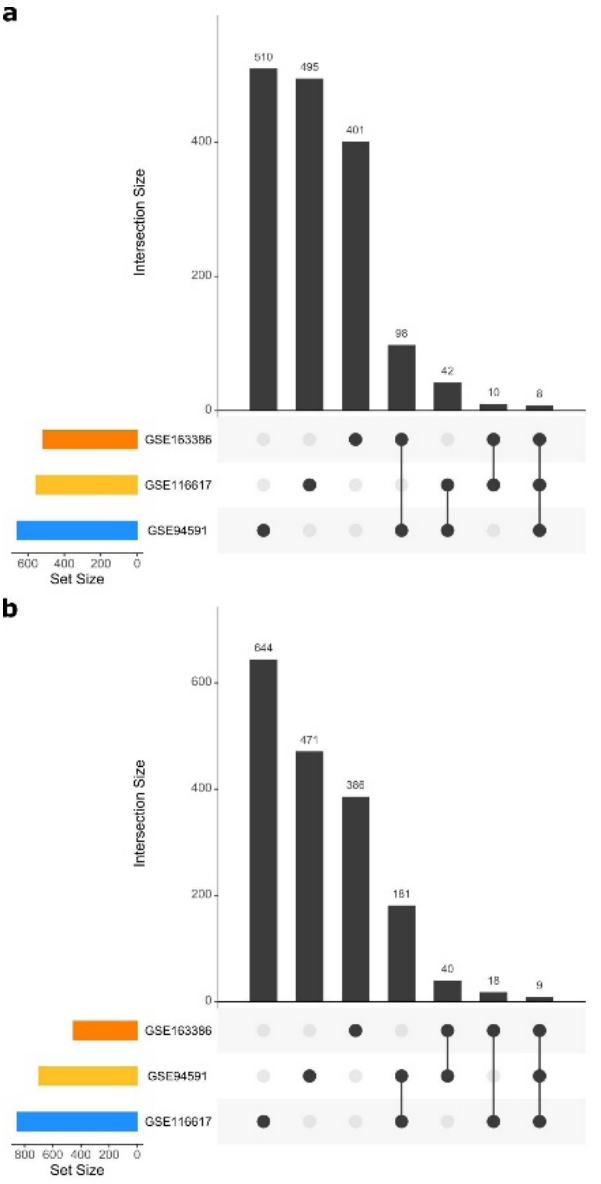

Figure 1. Dysregulated circRNAs detected in the GSE94591, GSE116617 and GSE163386 datasets. a) It was determined that 510 circRNA, 495 circRNA, and 401 circRNA were down-regulated in GSE94591, GSE116617, and GSE163386 datasets respectively. 98 circRNAs overlap in the GSE94591 and GSE163386 datasets. 42 circRNAs overlap in the GSE116617 and GSE94591 datasets. 10 circRNAs overlap in the GSE163386 and GSE116617 datasets. Eight circRNAs overlap in the GSE94591, GSE116617, and GSE163386 datasets. b) It was determined that 644 circRNA, 471 circRNA, and 386 circRNA were up-regulated in GSE116617, GSE94591, and GSE163386 datasets respectively. 181 circRNAs overlap in the GSE94591 and GSE116617datasets. 40 circRNAs overlap in the GSE94591 and GSE163386 datasets. 18 circRNAs overlap in the GSE163386 and GSE116617 datasets. Nine circRNAs overlap in the GSE163386, GSE94591, and GSE116617 datasets.

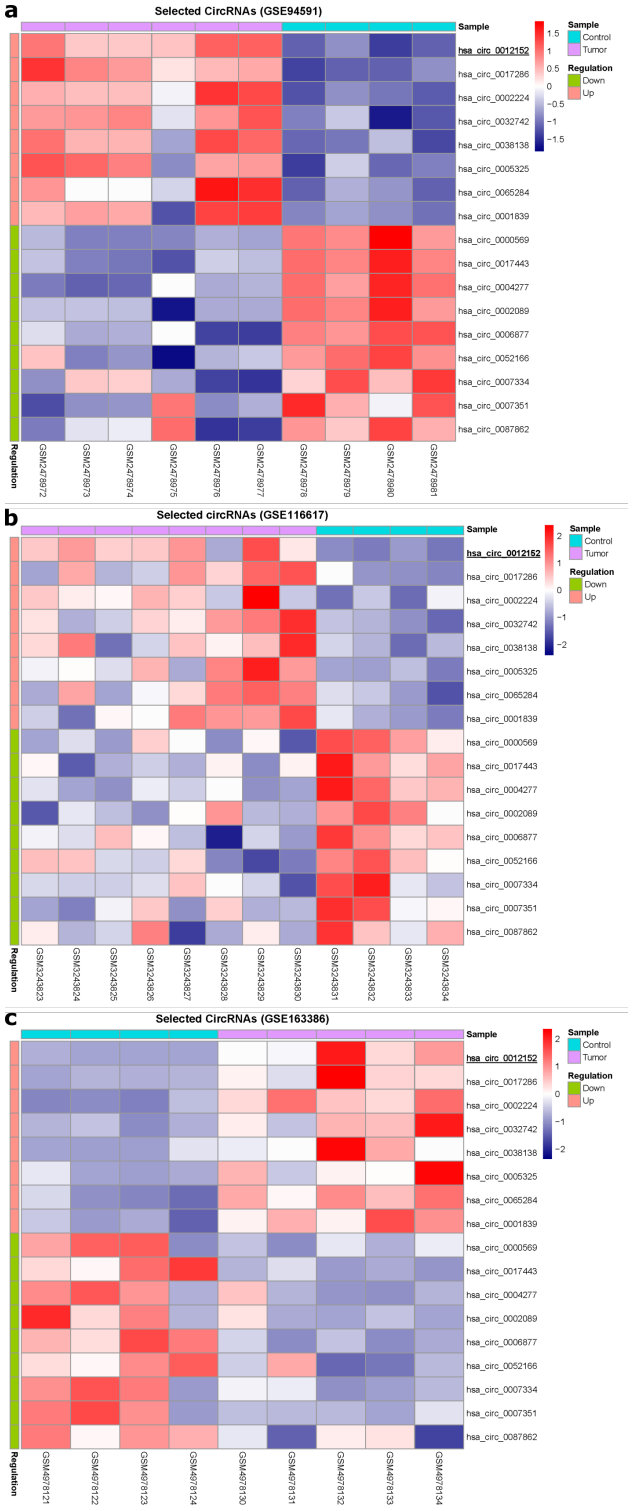

Figure 2. Heatmap of the overlapped down-regulated and up-regulated circRNAs a) GSE94591, b) GSE116617, c) GSE163386.

Survival Analysis

We utilized the gene expression profiling interactive analysis (GEPIA) program to determine the overall survival (OS) of the hub genes.

RESULTS

Detected Differentially Expressed circRNAs (DECs)

It was determined that there were 852 downregulated circRNAs and 555 upregulated circRNAs in the GSE116617 dataset, 453 downregulated circRNAs and 517 upregulated circRNAs in the GSE163386 dataset, and 701 downregulated circRNAs and 658 upregulated circRNAs in the GSE94591 dataset. Among these circRNAs, 9 downregulated and 8 upregulated circRNAs overlapped in 3 datasets (Figure 1). These 17 overlapping dysregulated circRNAs expression heatmaps are shown in Figure 2 for the GSE94591 (a), GSE116617 (b), and GSE163386 (c) datasets. As a result of the literature search, 2 downregulated circRNAs (hsa_circ_0002089, hsa_circ_0006877) and 2 upregulated circRNAs (hsa_circ_0012152, hsa_circ_0005325) were selected, which were determined to be closely related to AML and other cancers. Among them, hsa_circ_0012152 was considered to be more closely related to AML. Wilcoxon test results according to tumor and control samples are shown in Figure 3.

Detected Differentially Expressed miRNAs (DEMs)

It was detected that there were 46 downregulated miRNAs and 45 upregulated miRNAs in the GSE142699 dataset. Among these miRNAs, the top 10 downregulated and upregulated miRNAs were determined according to the log2FC value (Figure 4). After that, 2 downregulated miRNAs (miR-199a-5p, miR-376c-3p) that overlap with hsa_circ_0012152 and 1 downregulated miRNA (miR-495-3p), which overlaps with hsa_circ_0005325, were selected. The expression violin plot data of selected miRNAs are shown in Figures 5-a, b, and c and the volcano plot of the GSE142699 dataset is shown in Figure 5-d.

Detected Differentially Expressed mRNAs (DEGs) and Results of KEGG and GO Analysis

A total of 67 downregulated mRNA and 98 upregulated mRNA were detected in the GSE142698 dataset. The potentially targeted genes of these selected miRNAs were found by *in silico* tools; 33 genes for miR-199a-5p, 15 genes for miR-376c-3p, and 38 genes for miR-495-3p were identified. The circRNA–miRNA–mRNA regulatory network is shown in Figure 6a. The target genes' protein-protein interaction networks are shown in Figure 6b and 6c. According to the hub genes, the KEGG and GO pathway analysis results are shown in Figure 7.

Survival Analysis

Survival analysis of 10 possible target genes of miR-199a-5p was investigated. It was determined that Homeobox A9 (*HOXA9*) and Mediator of DNA damage checkpoint 1 (*MDC1*) genes may be important in the survival of AML patients (Figure 8).

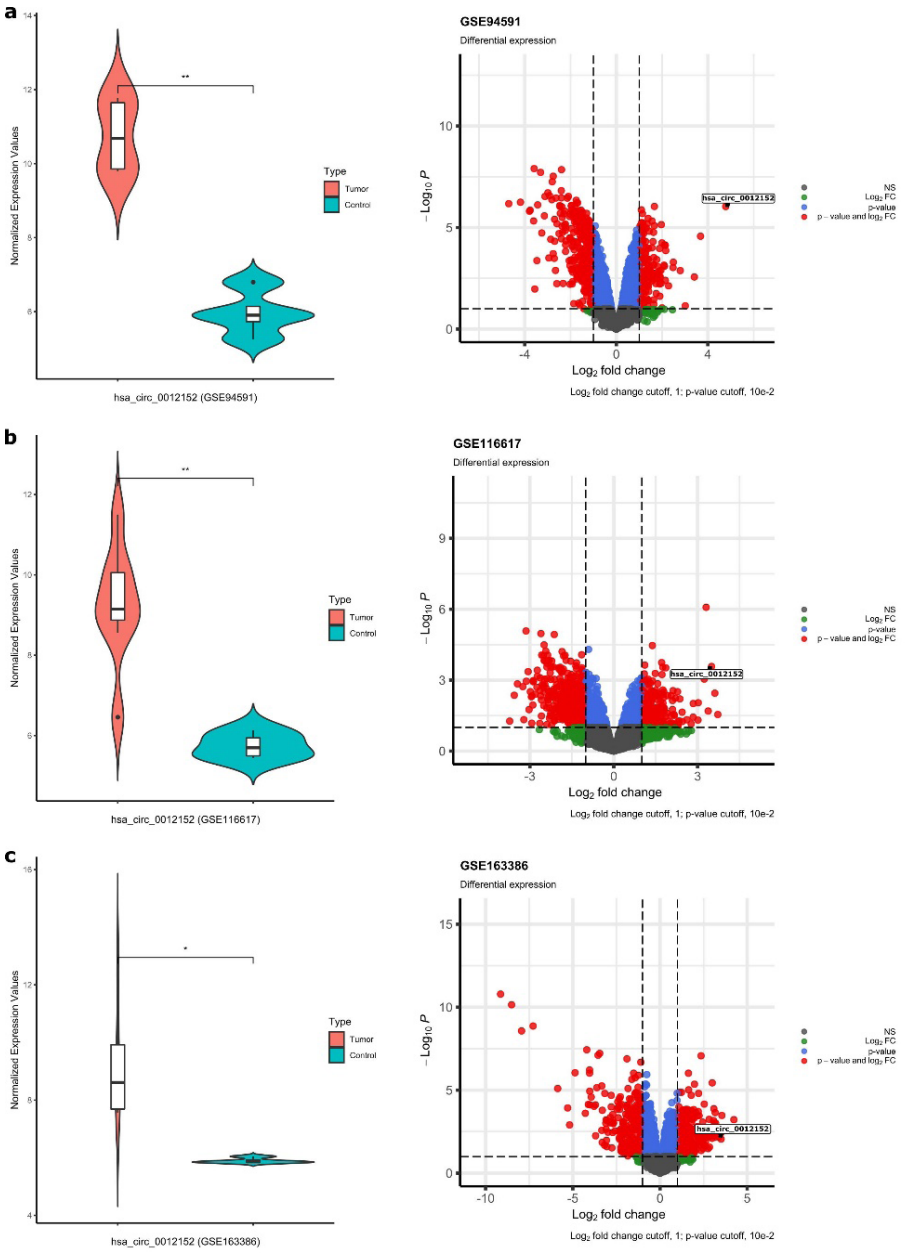

Figure 3. The violin and box plots of hsa_circ_0012152 in a) GSE94591 dataset, b) GSE116617 dataset, c) GSE163386 dataset.

DISCUSSION

Various microarray and RNAseq studies of circRNAs in AML have been performed and it has been determined that thousands of circRNAs were expressed differently in tumor tissue compared to normal tissue. Because it is difficult to study the complex link between these numerous circRNAs, miRNAs, and genes *in vitro and in vivo*, determining the most significant circRNA-miRNA-gene axis in "*in silico*" first will be advantageous. One of the most important reasons for this issue is that each circRNA has the potential to sponge so many miRNAs, and each miRNA has the power to change the expression level of hundreds of genes as well (12). Because of significant advances in bioinformatics, it is now possible to determine circRNA-miRNA-gene relationships *in silico*. Based on the findings of bioinformatics investigations, more precise results can be obtained in "*in vitro and in vivo*" studies.

The expression of hsa_circ_0012152, which we detected to be up-regulated in all 3 datasets in our bioinformatics study, was reported to be up-regulated in AML samples according to both microarray and qRT-PCR results in the study performed by Guo S et al. Moreover, in the same study, it was emphasized that hsa_circ_0012152 may have an expression pattern that distinguishes between AML and ALL (13). It has been reported that hsa_circ_0005325, another prominent circRNA in our

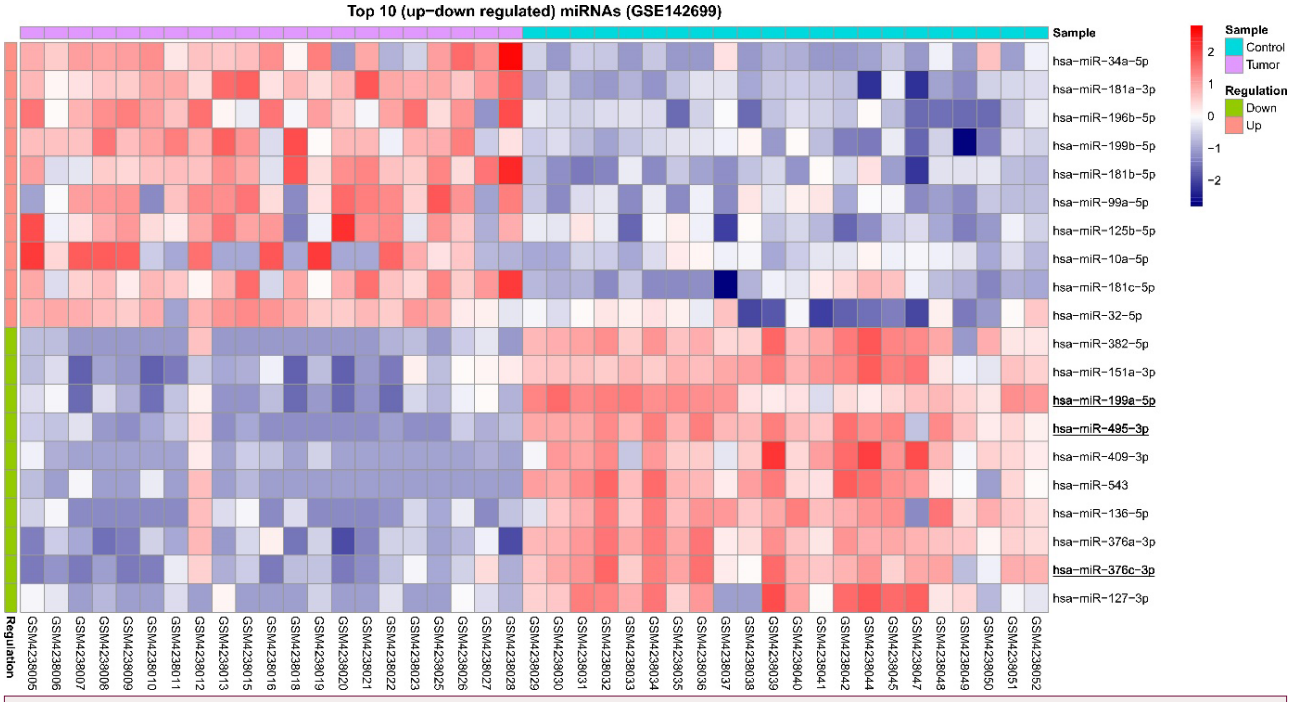

Figure 4. GSE142699 heat map of top 10 selected miRNAs.

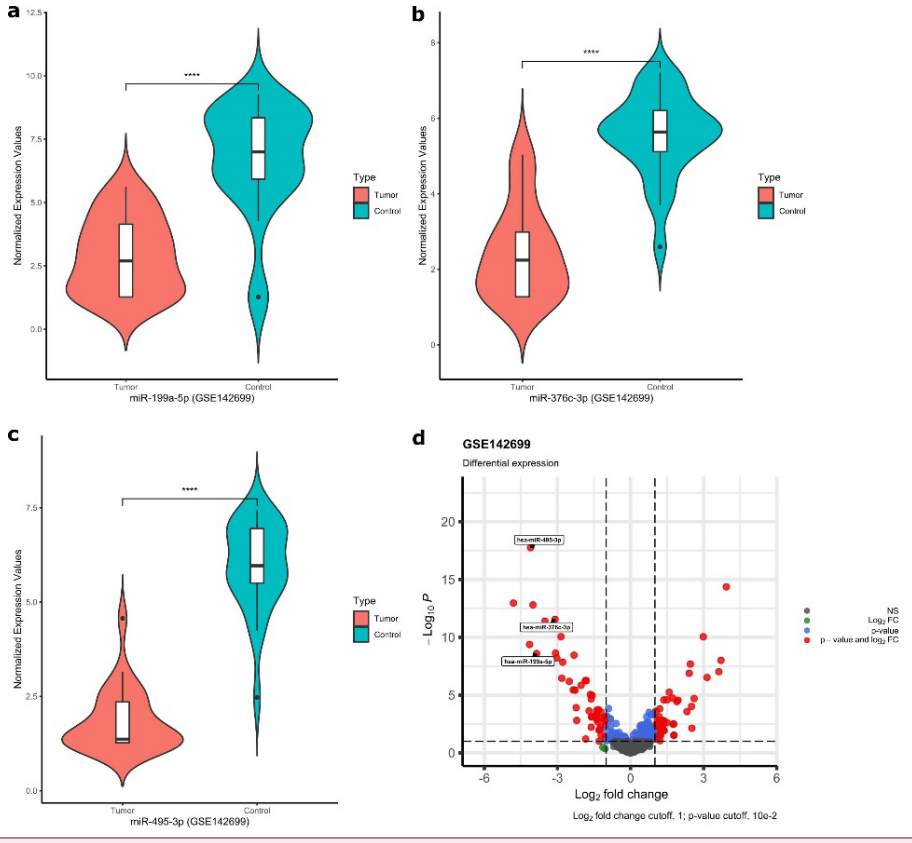

Figure 5. Expression violin plot data of selected miRNAs a) miR-199a-5p, b) miR-376c-3p, c) miR-495-3p, d) GSE142699 volcano plot.

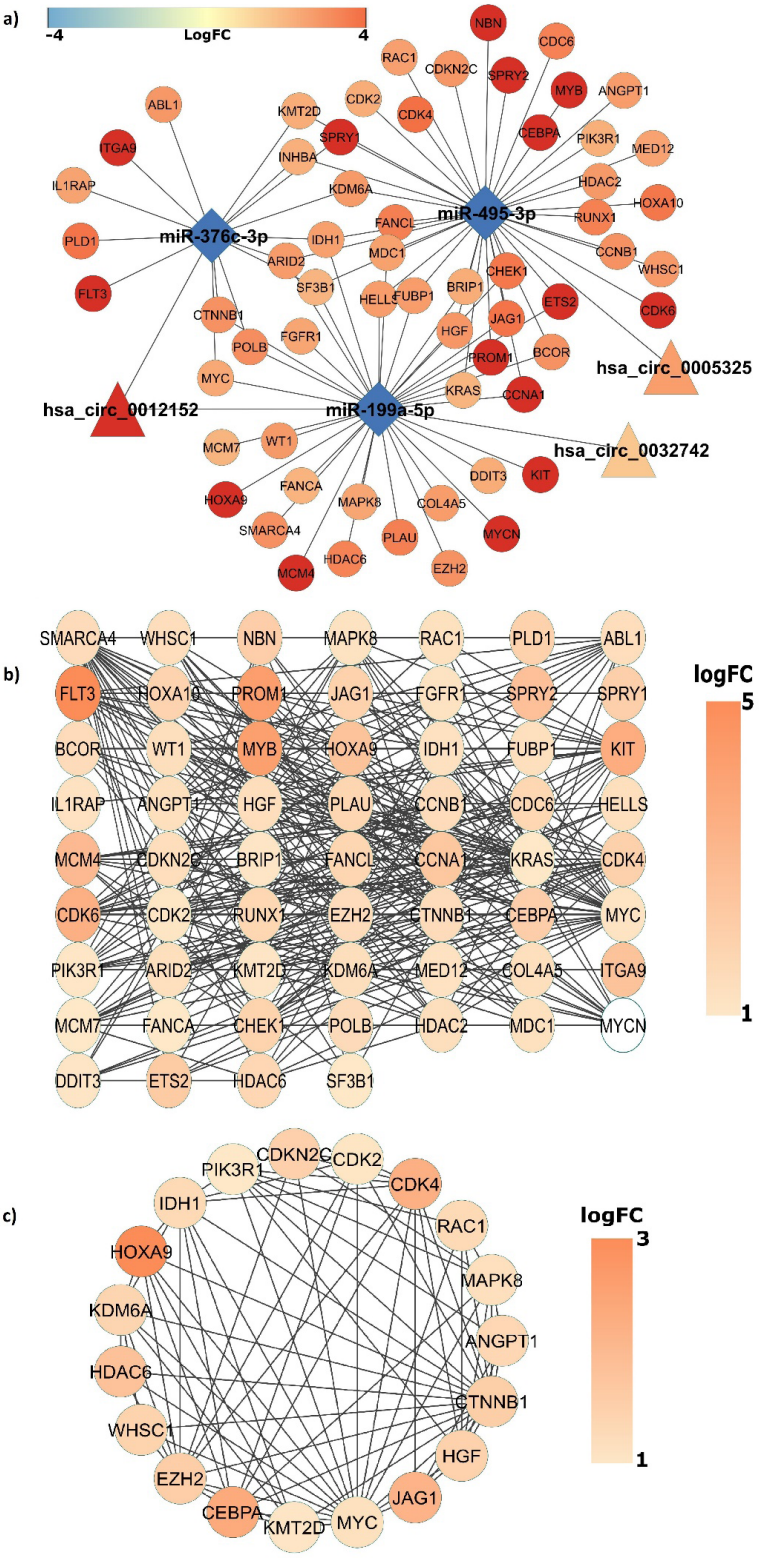

Figure 6. circRNA–miRNA–mRNA regulatory network and protein-protein interaction network of the target genes. a) The network consisting of three cricRNAs (hsa_circ_0012152, hsa_circ_0005325, and hsa_circ_0032742), three miRNAs (miR-376c-3p, miR-495-3p, and miR-199a-5p), and 62 genes was generated by Cytoscape 3.9.0. b) PPI network of the 60 target genes that exert momentous roles in AML. c) The hub genes were identified by the MCODE tool.

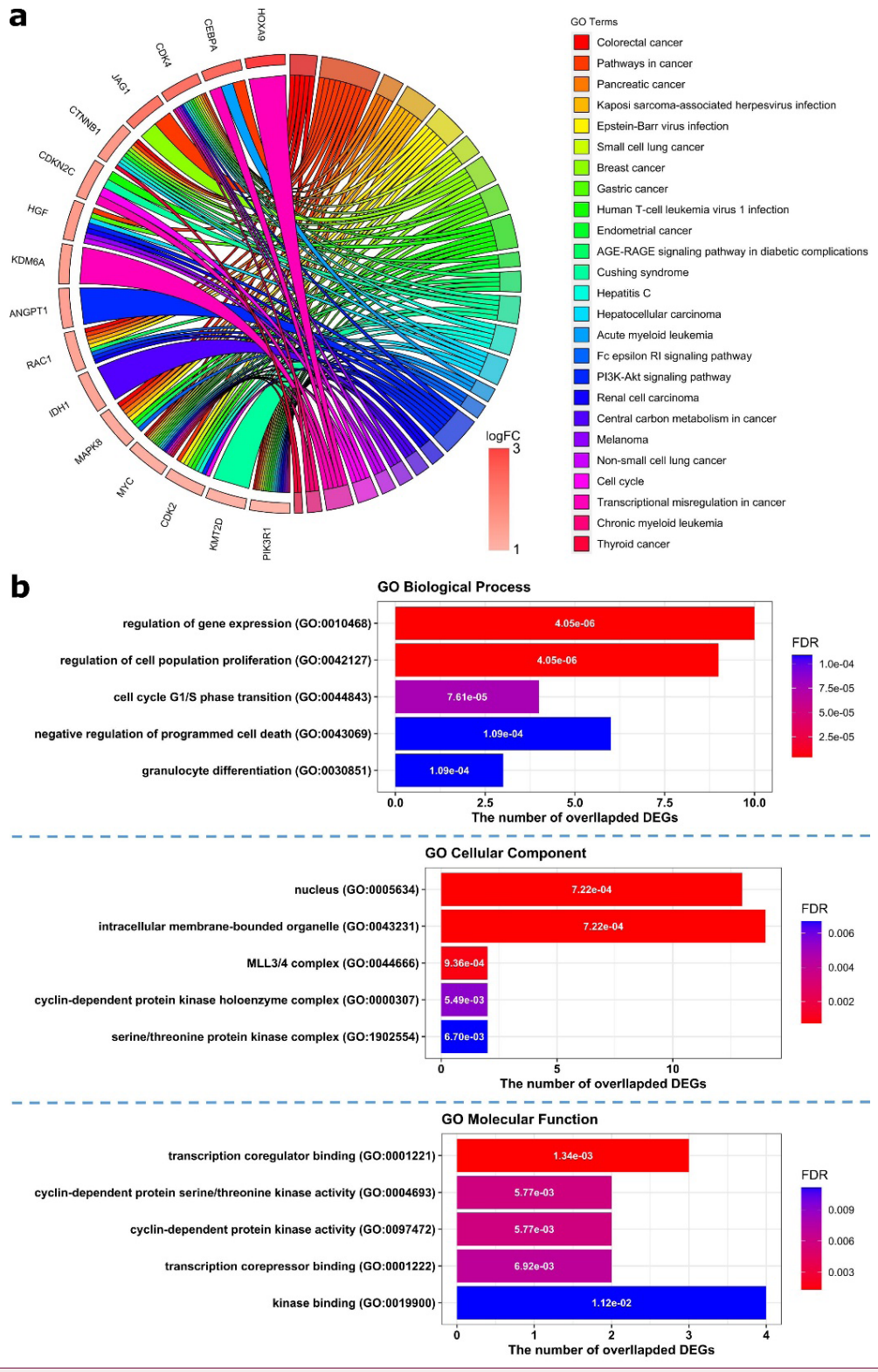

Figure 7. KEGG and GO pathway analysis results. a) The significantly enriched KEGG pathways with a FDR < 0.05. Cohort plot shows that the sixteen hub genes are correlated via ribbons with their assigned KEGG terms. b) Top five GO enrichment annotations of the sixteen hub genes (biological process, cellular component, molecular function). FDR is calculated using the Benjamini-Hochberg method to adjust the multiple hypothesis testing. KEGG: Kyoto encyclopedia of hub-genes and genomes; GO: Gene ontology; DEGs: Differentially expressed genes; FDR: False discovery rate.

study, is also upregulated in lung adenocarcinoma cells (14) and hepatic fibrosis (15). It has been shown that hsa_circ_0002089, which was detected to be significantly down-regulated in our AML datasets, is also among the top 10 down-regulated circRNAs in gastric cancer (16). hsa_circ_0006877 (circLDLR), which was found to be decreased in AML in our study data, has also been shown in the literature to be associated with various cancers such as papillary thyroid carcinoma and hepatocellular carcinoma (17, 18). In addition, it has been demonstrated that the hsa_circ_0006877 expression level decreases in some diseases other than cancer, such as polycystic ovary syndrome(19). Of these 4 circRNAs with significant expression changes in all 3 datasets, hsa_circ_0012152 and hsa_circ_0005325 were also found to interact with the miRNAs detected by the analysis of the GSE142699 dataset in our study. We also determined that hsa_circ_0005325 could be the sponge for miR-495-3p. In the literature, the LGMN pseudogene has been reported to promote tumor progression by acting as a sponge for miR-495-3p in glioblastoma cancer (20). In AML, it has been reported that miR-495-3p may have a pivotal role in patients with cytogenetically normal (21). It has been reported that miR-376c-3p, one of the two miRNAs determined to interact with hsa_circ_0012152, may affect the cell cycle in neuroblastoma cells via cyclin D1 (22). It has been shown that miR-199a- 5p may have a crucial role in many cancer types, including AML. For example, miR-199a-5p was sponged by Linc00662 in bladder cancer and its role in tumor development has been reported (23). In another study, it was shown that this miRNA is involved in the regulation of cancer stemness via the HOTAIR/Sp1 axis in cutaneous-squamous cell carcinoma (24). It has been shown that miR-199a-5p is effective in the sensitivity of AML cells to Adriamycin via the *DRAM1* gene (25). It has been stated that miR-199a-5p plays a role in the regulation of the chemoresistance process, and it has been emphasized that it may be an important therapeutic target miRNA in drug-resistant AML (26). Many of the 33 genes in the GSE142699 dataset identified as targets of miR-199a-5p have been reported to be associated with cancer and AML. For example, the *MDC1* gene, which was determined as the hub gene among these genes, was found to be closely related to many cancers (27, 28). In the study by Ruff et al., it was suggested that *MDC1* may be an important biomarker in carcinogenesis (29). Another hub gene, *HOXA9*, which is among the important targets of the miR-199a-5p, has been reported as a director of the prognosis of the disease by playing a role in the increase of blood cells in AML (30).

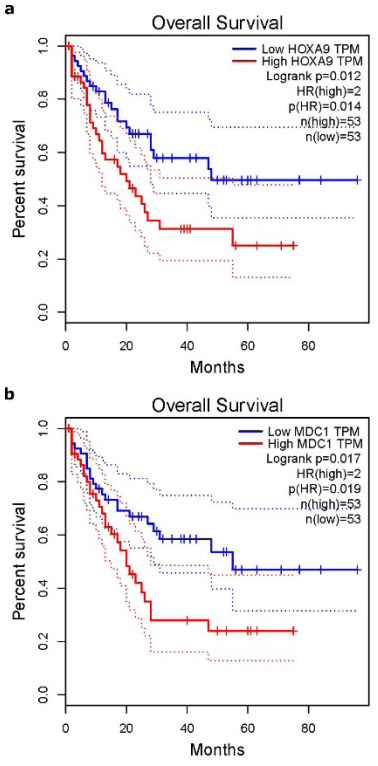

Figure 8. Survival analysis of the *HOXA9* (a) and MDC1 (b) in AML patients.

CONCLUSION

In this study, a meta-analysis of circRNA, miRNA, and mRNA datasets in AML was performed with bioinformatics tools and circRNA-miRNA-mRNA axes that may be important in AML were determined. As a result of all *in silico* evaluations and a detailed literature review, it was understood that hsa_circ_0012152, miR-199a-5p and *HOXA9* may be important for AML. In summary, it was thought that the role of the hsa_circ_0012152/miR-199a-5p/*HOXA9* axis in AML should be investigated with further studies *in vitro and in vivo*.

Ethics Committee Approval: The results of the study were obtained using public Geo Datasets. Since these data are bioinformatics analysis data and there is no clinical or experimental studies have been conducted, ethics committee approval is not required.

Author Contributions: Conception/Design of Study- C.E., M.K., I.S.; Data Analysis: C.E.; Interpretation and Drafting Manuscript- I.S., M.K.; Critical Revision of Manuscript- C.E., M.K., I.S.; Final Approval – C.E., M.K., I.S.

Conflicts of Interest: The authors declare no conflict of interest.

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Association of EGFR Gene Polymorphism with Glioma Susceptibility in Turkish Population

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ABSTRACT

Objective: Gliomas are devastating adult brain tumors of unknown etiology, occupying 8 out of 10 primary brain tumors. Epidermal growth factor receptor (EGFR) as a tyrosine kinase family member is encoded by the EGFR gene located in chromosome 7p12-13. Various studies have identified numerous SNPs, including those in the EGFR gene, as linked to gliomas. The objective of the investigation was to determine whether the genotype and allele frequencies of the EGFR may have a role in glioma susceptibility.

Materials and Methods: To examine the association of EGFR SNP rs1468727 with glioma susceptibility in a case-control study from Türkiye (34 cases, 36 controls), genotyping and statistical analyses were performed by using real-time-polymerase chain reaction (RT-PCR) and SPSS version 25.0, respectively.

Results: A significant relationship was found between the study groups EGFR SNP rs1468727 genotypes (p = 0.028). The CC genotype frequency was significantly greater in the control group compared to the glioma group (p=0.005). When compared with the control group, the frequency of mutant type T allele carriers was significantly higher in glioma patients (p=0.012).

Conclusion: As a result of the preliminary findings, having the mutant T allele may increase risk by 3.36 times, whereas having the ancestral homozygote CC genotype lowers the risk for glioma in Turkish population.

Keywords: Glioma, EGFR gene, variation, SNP

INTRODUCTION

Gliomas, which account for 3 out of 10 of all brain tumors and 8 out of 10 malignant brain tumors, are adult tumors that develop from the neuroglia, the brain's support cells (1). They encompass malignant brain tumor groups of varying degrees, in which genetic factors affect the development and progression of the disease, and patients have a very short life expectancy after diagnosis (1- 3).

Most gliomas are the result of inherited genetic variations intertwined with environmental factors. Due to this, the roots of the evolution of gliomas can be grouped under two major headings. One is genetic factors like single nucleotide polymorphisms (SNPs), and another is cooperation among environmental elements like lifestyle habits and comorbidities (2,4). Epidemiological studies have identified numerous SNPs, including those in the epidermal growth factor receptor (EGFR) gene, as linked to gliomas (3,5,6).

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One of the ErbB receptor family members, EGFR, is responsible for inducing the proliferation to the survival of different cell types as a product of chromosome 7's short arm, plays a role in the pathogenesis of various types of cancer such as nearly half of the malignant gliomas (6,7). Further, the EGFR pathway joins into cellular processes through a progression of the cell cycle to angiogenesis, which also prepares the basis for tumorigenesis (6). The external ligand-binding region of the EGFR is activated when a ligand binds to it. This causes the receptor to dimerize and becomes tyrosine auto-phosphorylated, which activates the receptor (8). Multifarious biological processes, from somatic mutations to gene amplification, underlie the changes in EGFR gene expression. Among gliomas, both high expression and amplification of the EGFR gene are distinctive for primary glioblastoma (9). Also, alterations in the expression of this gene are known as a causative factor for resistance to treatments such as radiation and chemotherapy. Due to this feature, anti-EGFR therapies have become a treatment strategy. However, treatment strategies are aiming to rule over EGFR activation seem to be interrupted in the glioblastoma subtype (10,11). It is essential to comprehend how variations in EGFR's structure affect the development and progression of glioma types since EGFR plays a significant role in the molecular biology of gliomas. This knowledge is necessary to create effective treatment strategies.

Numerous SNPs have been reported in different genes in gliomas (4). A few of these reported SNPs (rs1052576, rs55705857, and rs17577) have been investigated to find if they are associated with gliomas, in studies performed in the Turkish population (12-15). According to human genome studies, EGFR gene SNPs may both be protective against glioma and offer a risk for it. Various populations have been studied for EGFR SNPs including rs1468727, rs730437, rs2252586, rs11979158, and rs11506105 (6). On the other hand, no studies have been performed to determine whether EGFR SNPs are linked to gliomas in the Turkish population. Although several studies have demonstrated the linkage between EGFR SNPs and gliomas, it is unclear which EGFR SNPs can serve as glioma biomarkers.

Considering the previously stated information, understanding EGFR, which contributes to tumor development by participating in processes such as metastasis , and by alternations due to different genetic and/or environmental factors, will enable the nature of gliomas to be clarified. In addition, due to the highly heterogeneous character of gliomas, examining whether it shows a population-based difference in its origin demonstrates a path for new treatment approaches. With this knowledge, this was the first Turkish population-based investigation that aimed to present a different perspective on the molecular biology of glioma by appraising the relationship between EGFR SNP rs1468727 and glioma.

MATERIALS AND METHODS

Study Population

Blood samples for this case-control study were obtained from glioma patients aged 18-85 (n = 34) and healthy individuals (n = 36) selected by the Department of Neurosurgery of Yeditepe University Hospital (Istanbul, Turkiye). This study was carried out in conformity with the principles of the Declaration of Helsinki and was approved by the Yeditepe University Faculty of Medicine's ethical committee with decision number 1757.

Genetic Analyses

The DNA of the study groups was extracted from peripheral blood samples that were kept in 5ml EDTA-coated tubes at +4°C until the analysis. An iPrep DNA extraction robot and Invitrogen iPrep Pure Link gDNA Blood Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) were utilized to perform the DNA isolation. With the help of NanoDrop 2000 (Thermo Scientific, Waltham, Massachusetts, USA), DNA samples were measured.

The real-time-polymerase chain reaction (RT-PCR) instrument 7500 Fast (Applied Biosystems, Foster City, CA, USA) was used. This system and TaqMan Assay were used in genotyping for EGFR SNP rs1468727. F: 5'GATCCAGAAATTTAGGAGC3' and R: 5' TTTCATCACCTTGCCTCT3' were specifically designed primers for the EGFR gene (rs1468727) polymorphism (Applied Biosystems, Foster City, CA, USA).

The phases of RT-PCR after holding the stage at 95°C for ten minutes were as follows: denaturation 40 cycles at 92°C for fifteen seconds, binding/elongation step 40 cycles at 60°C for one minute.

Statistical Analyses

All data were analyzed using SPSS version 25.0. Student's t-test was utilized to evaluate the difference between healthy individuals, patients with glioma. χ^2 and Fisher's exact tests were used to analyze the EGFR SNP rs1468727 in the study population. p values that were less than 0.05 were considered significant.

RESULTS

Demographic Profile of the Study Population

In this study, genotyping in blood samples was completed in seventy individuals. Of the thirty-four glioma cases, 9 (26.48%) were female, 25 (73.52%) were male. The control group consisted of 13 females (36.12%) and 23 males (63.88%) (Table 1). The mean age (p = 0.138) and gender (p = 0.385) of the patients with glioma and healthy controls were not significant.

Genotyping

The allelic and genotypic information of the study groups are provided in Table 2. The frequencies of CC:CT:TT genotypes were 19:16:1 in healthy controls and 8:22:4 in glioma patients. As a result of our analyses, the frequency of the EGFR SNP rs1468727 was significantly different between the patients with glioma and healthy controls (p = 0.028). The genotype

distribution p values were as follows: the CC homozygous wild type (p = 0.005), CT heterozygous (p = 0.089), and TT homozygous mutant (p = 0.145).

Table 1. Demographic characteristics of patients and control groups.

Parameters	Patient (n=34)	Control (n=36)	p value
Gender (%)	Male / Female 73.52 / 26.48 (n=25) / (n=9)	Male / Female 63.88 / 36.12 (n=23) / (n=13)	0.385
Age (years, mean± SD)	48.29±18.46	42.75±11.70	0.138

The CC genotype was observed at lower frequency (p = 0.005, odds ratio (OR) = 0.232, 95% confidence intervals (CI) = 0.081-0.068), and T allele was found to be higher (p = 0.012, OR = 3.632, 95% CI = 1.300-10.151) in the patients with glioma. In both EGFR CT and TT genotype samples, there were no significant differences between the study groups (p = 0.089 and p = 0.145, respectively). Also, the EGFR ancestral C allele frequency was not statistically significant (p = 0.145). These results can be interpreted such that carrying T allele variant increased the glioma risk (p = 0.012, OR = 3.632, 95% CI = 1.300-10.151), and carrying CC homozygote genotype decreased the disease risk (Table 2).

DISCUSSION

Despite the current acceleration of genome research, particularly in polymorphism studies, there is still a great deal of uncertainty regarding the genetic background of glioma susceptibility. Therefore, large-scale patient-control studies to be carried out in different populations are of great importance in clarifying this molecular issue.

As a gene that encodes a transmembrane receptor tyrosine kinase and is localized on chromosome 7p12-13, EGFR is known to be involved in cell processes that are at the core of cancer research (16). Prior studies on activation of the EGFR signaling and genetic changes in its genes have reported the relationship of these changes with carcinoma development (17, 18). Especially for glioblastoma, changes in EGFR expression are more important and associated with poor outcomes (19). Amplification of EGFR caused by genetic alterations was discovered to be a type of pathological genetic change in more than 40% of gliomas (20, 21).

Molecularly targeted studies are necessary for the well-rounded treatment of cancer. Genetic investigations have been performed on human gene polymorphisms such as the EGFR SNPs in different populations and different types of cancers to identify their potential role in the diseases. Therefore, we investigated the SNP rs1468727 of the EGFR gene in a Turkish population. This SNP may be linked to factors that lead to cancer progression by allowing cells to grow and by causing changes in the activation of receptors, expression, or stability of EGFR.

The results of this study showed a link between EGFR SNP rs1468727 and glioma. In patients within the glioma group, the CC genotype was significantly lower; however, the T allele was found to possibly contribute to glioma development. Population studies have determined a link between glioma development and EGFR SNPs; a Chinese population-based study reveals the association of EGFR SNP rs1468727 with susceptibility to glioma. Li et al. have shown that the CC genotype may increase the risk of glioma (6). Furthermore,

Table 2. Genotype and allele frequencies between patients with glioma and the healthy controls.

Polymorphism	Glioma % (n)	Control % (n)	p value	Odds Ratio	95% CI
EGFR (rs1468727)	n=34	n=36	**0.028***		
CC	23.5 (8)	52.8 (19)	**0.005***	0.232	0.081-0.068
CT	64.7 (22)	44.4 (16)	0.089	2.292	0.875-6.0002
TT	11.8 (4)	2.8 (1)	0.145	4.667	0.494-44.051
	Allelic count	Allelic count			
C	55.88 (38)	75 (54)	0.145	0.214	0.023-2.023
T	44.12 (30)	25 (18)	**0.012***	3.632	1.300-10.151

*statistically significant
n: number of observation, OR: odds ratio, CI: confidence interval.

they explained that the reason for such a result is that SNP rs11468727 may increase cell proliferation by causing a change in EGFR activation (6). Also, Hou et al., obtained the same results to confirm other studies on the Han Chinese Population; the CC of this SNP was pointed out as a risk marker (22). Other studies in Chinese populations showed that the C allele (23) and the CC genotype (24) elevated the disease risk, which was consistent with other results obtained in the aforementioned studies. Also, Yu et al. stated that rs1468727 is a genetic factor that increases the risk of glioma in the Asian population (25). In addition to these results, the EGFR SNP rs1468727 TT genotype was reported as protective from glioma (26).

On the other hand, Andersson et al. and Baek et al. have investigated the effect of several SNPs in European and Korean populations with glioma. They could not validate that EGFR SNP rs1468727 affects glioma development and/or progression in both populations (27, 28).

According to the literature review, this case-control study is the first investigation to address the EGFR SNP rs1468727 as a glioma-related indication in the Turkish population. Our study revealed that EGFR gene polymorphism rs1468727 could affect glioma development. The outcomes showed that carrying EGFR SNP rs1468727 homozygous wild-type may be a risk-reducing factor ($p = 0.005$), whilst carrying the T allele may increase the risk for glioma ($p = 0.012$). These observations can be interpreted such that an intronic SNP rs1468727 may interfere with EGFR expression and have a role in glioma genetics.

As expected, different results could be reported due to variables such as ethnic differences and increasing sample sizes. Since there is a significant relationship between EGFR SNP rs1468727 and glioma, it seems to be an important gene region for elucidating the molecular mechanism of the disease. In addition, because it is a polymorphism in the intronic region, which gene regions it interacts with is another issue that needs to be investigated.

Limitations

The small sample size was a major limitation of the study. Future investigations with a larger sample size would be helpful to confirm these preliminary findings.

CONCLUSION

Consequently, this study is the first to suggest that EGFR SNP rs1468727 is associated with glioma in the Turkish population. While carrying the CC genotype appears to be a protective factor, the T allele might be a genetic marker for the risk of glioma.

Ethics Committee Approval: All procedures performed in studies involving human participants were made under the ethical standards of the 1975 Declaration of Helsinki guidelines and its later amendments. The research on humans study protocol was approved by the Yeditepe University Medical Faculty Ethics Committee (file no: 21.11.2019/1757).
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Impact of Anogenital Distance Parameters on Female Sexual Dysfunction

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ABSTRACT

Objective: The aim of this study was to investigate the relation between anogenital distance (AGD) and female sexual dysfunction.

Materials and Methods: The present study was done prospectively between January 2021 - July 2022. All patients filled out the Female Sexual Function Index (FSFI) score and the Sexual Quality of Life-Female score (SQOL-F). Also, AGD was measured in all patients. Patients were classified into two groups according to FSFI (FSFI <27 and FSFI ≥27) and into three groups according to SQOL-F (SQOL-F 18-51, SQOL-F 52-84, SQOL-F >84). Groups were compared according to age, body mass index (BMI), parity status, anogenital anoclitoral distance (AGD_{AC}), anus to fourchette distance (AGD_{AF}), and genital hiatus (GH). Also, correlation analysis was performed between sexual function scores and AGD.

Results: Totally, 280 patients were enrolled into the study and 89 (31.8%) patients had sexual dysfunction according to FSFI. AGD_{AC} (74.7 mm vs 64.6 mm, p= 0.001) and GH length (27.8 mm vs 22.0 mm, p= 0.001) were significantly longer in patients with sexual dysfunction. In addition, GH and AGD_{AC} were significantly shorter in patients with the highest SQOL-F. Correlation analysis showed no significant correlation between AGD_{AF} and sexual function (p= 0.671 for FSFI and p=0.294 for SQOL-F). However, longer AGD_{AC} was significantly and negatively correlated with healthy sexual status (r= - 0.546, p= 0.001 for FSFI and r= - 0.604, p= 0.001 for SQOL-F). In addition, longer GH distance was significantly associated with female sexual dysfunction (p= 0.001 for FSFI and p= 0.001 for SQOL-F).

Conclusion: The present study demonstrated that almost one third of women had sexual dysfunction. Also, the present study found that longer AGD_{AC} and GH were significantly associated with female sexual dysfunction and female sexual dissatisfaction according to FSFI and SQOL-F for the first time.

Keywords: Anogenital distance, genital hiatus, female sexual dysfunction, FSFI, SQOL-F

INTRODUCTION

Female sexual dysfunction is accepted as a clinically significant inconvenience in sexual relations which is associated with personal distress. It is well known that an unsatisfactory sex life may result in depression, loss of self-confidence, and deterioration of the relationship with a partner (1,2). For many years, discussing predictive factors and solutions for female sexual dysfunction has been considered taboo, and female sexual dysfunction has been overlooked. However, studies conducted towards the end of the 20th century showed that almost 2 out of every 5 women suffer from sexual dysfunction (3). Predictive factors for female sexual dysfunction are one of the hottest topics in gynecology, and previous reports investigated many factors that may play a role in female sexual dysfunction, including menopausal status, surgical history, and anatomical factors (4).

Anogenital distance (AGD) is an anatomical landmark which describes the distance between the anus and external genitalia. AGD has been the subject of many studies, and the effect of AGD on prostate cancer, polycystic ovary, endometriosis, incontinence, and

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premature ejaculation has been investigated (5). Toprak et al. analyzed AGD in patients with premature ejaculation, and AGD was found to be significantly longer in patients who suffered from premature ejaculation (6). Also, Sánchez-Ferrer and colleagues stated that increased genital hiatus (GH) length and anogenital anoclitoral distance (AGD_{AC}) played a role in pelvic prolapse in females (7). In addition, some authors have concluded that stress urinary incontinence is significantly more common in women with longer AGD_{AC} and shorter anus to fourchette distance (AGD_{AF}) (8).

While previous studies investigated the impact of AGD on some female diseases, no study has analyzed the correlation between AGD and female sexual function. The aim of the present study was to investigate the relation between AGD and female sexual dysfunction.

MATERIALS AND METHODS

The present study was done prospectively between January 2021 – July 2022, and 280 woman who were admitted as gynecology outpatients were evaluated for inclusion in the study. All participants signed informed consent. Ethics committee approval was obtained from the Istanbul Haseki Training and Research Hospital ethics committee (2020-124). The medical history of patients was recorded, and a physical examination was performed for all participants. All patients completed the Sexual Quality of Life-Female score (SQOL-F) and the Female Sexual Function Index (FSFI) score under the supervision of a physician. Also, AGD was measured in all patients. Patients were classified into two groups according to FSFI (FSFI <27 and FSFI ≥27) and into three groups according to SQOL-F (SQOL-F 18-51, SQOL-F 52-84, SQOL-F >84). Patients who were not able to fill out the FSFI or SQOL-F, patients with severe psychiatric disease, patients with endocrine disorders, pregnant patients, patients in the postpartum period, patients in menopause, and patients abstaining from sexual intercourse were excluded from the study. Other exclusion criteria were being <18 years old, history of perineal surgery, and infectious conditions in the anogenital area.

Patients' demographic information were noted. The AGD was measured by digital caliper (Supplier: VWR® International, LLC, West Chester, PA, USA) in the lithotomy position. Length from the upper edge of the anus to the clitoris was defined as AGD_{AC}. Linear measurement between the upper edge of the anus and fourchette was AGD_{AF}. GH length was measured between the center of the urethral meatus and perineum nucleus. To prevent incorrect or erroneous measurements, two physicians measured each parameter mentioned above.

FSFI and SQOL-F score

The FSFI is a survey, containing 19 questions, used for evaluation of female sexual function. The questionnaire gives information about arousal, desire, orgasm, pain, lubrication, and satisfaction, and the FSFI scored from 2 to 36 for each patient. An FSFI score <26 is related to female sexual dysfunction (9). The SQOL-F is a self-reported questionnaire containing 18 items. Each question is scored from 1 to 6 or 0 to 5 and the total SQOL-F score ranges from 18 to 108 (worst to best) (10).

To understand the impact of AGD on sexual function, patients were categorized into two groups according to FSFI (FSFI <27 and FSFI ≥27) and into three groups according to SQOL-F (SQOL-F 18-51, SQOL-F 52-84, SQOL-F >84). Groups were compared according to age, body mass index (BMI), parity status, AGD_{AC}, AGD_{AF}, and GH. Also, correlation analysis was performed between sexual function scores and AGD.

Statistical Analysis

The Statistical Package for the Social Sciences version 25 (SPSS IBM Corp., Armonk, NY, USA) program was used. The independent Student's t test was performed to analyze normally distributed parameters, and the Mann-Whitney U test was used to evaluate non-normally distributed data. The one-way ANOVA test and the Kruskal Wallis test were used for continuous parameters. The relationships between AGD, FSFI, scores and SQOL-F scores were evaluated with bivariate correlation analysis. Categorical variables were analyzed using the $\chi 2$ test. A p value of less than 0.05 was defined as statistically significant.

RESULTS

In total, 280 patients were enrolled into the study. The mean age and mean BMI of participants were 31.5 years and 27.0 kg/m^2, respectively. A total of 17.5% (49 women) were nulliparous. The mean AGD_{AF}, AGD_{AC}, and GH distances were 24.5 mm, 67.8 mm, and 23.8 mm, respectively. Also, mean FSFI and mean SQOL-F were 29.3 and 65.5 for all patients (Table 1).

Table 1. Characteristics of patients.

	n=280
Age (years)*	31.5±7.4
BMI (kg/m^2)*	27.0±4.8
Parity n; (%)	
Nulliparous	49 (17.5%)
Parity ≥1	231 (82.5%)
AGD from the anus to the fourchette (mm)*	24.5±6.1
AGD from the anus to the clitoris (mm)*	67.8±9.9
GH (mm)*	23.8±5.7
FSFI score*	29.3±5.4
SQOL-F score*	65.5±23.4

*Mean ± standard deviation; AGD: Anogenital distance; GH: Genital hiatus; FSFI: Female sexual function index; SQOL-F: Sexual Quality of Life-Female score.

Table 2. Demographic data and AGD according to FSFI score.

	FSFI score <27 (n:89)	FSFI score ≥27 (n:191)	p value
Age (years)*	32.3±6.6	31.1±7.7	0.649
BMI (kg/m^2)*	26.8±4.9	27.1±4.7	0.327
Parity n; (%)			
Nulliparous	19 (21.3%)	30 (15.7%)	0.637
Parity ≥1	70 (78.7%)	161 (84.3%)	
AGD_{AF} (mm)*	24.9±5.4	24.3±6.4	0.388
AGD_{AC} (mm)*	74.7±8.2	64.6±9.0	***0.001***
GH (mm)*	27.8±6.7	22.0±4.2	***0.001***

*Mean ± standard deviation; AGD_{AC}: Anogenital distance from the anus to the clitoris, AGD_{AF}: Anogenital distance from the anus to the fourchette, BMI: Body mass index, GH: Genital hiatus, FSFI: Female Sexual Function Index.

Eighty-nine (31.8%) patients had sexual dysfunction according to FSFI. Comparison of the groups according to FSFI revealed that age, BMI, parity status, and AGD_{AF} were comparable (p= 0.649, p= 0.327, p= 0.637, and p= 0.388). However, AGD_{AC} (74.7 mm vs 64.6 mm) and GH length (27.8 mm vs 22.0 mm) were significantly longer in patients with sexual dysfunction (p= 0.001 and p= 0.001) (Table 2). In addition, 77 women had SQOL-F between 18-51, 120 women had SQOL-F between 52-84, and 83 women had SQOL-F >84, respectively. Age, BMI, parity status, and AGD_{AF} were similar between groups according to SQOL-F. AGD_{AC} and GH were significantly shorter in patients with the highest SQOL-F (Table 3).

Correlation analysis found no significant outcome between AGD_{AF} and sexual function scores (p= 0:671 for FSFI and p= 0.294 for SQOL-F). Longer AGD_{AC} was significantly negatively correlated with healthy sexual status (p= 0.001 for FSFI and p= 0.001 for SQOL-F). Longer GH distance was significantly associated with female sexual dysfunction (p= 0.001 for FSFI and p= 0.001 for SQOL-F). Correlation analysis for AGD and sexual function scores are summarized in Table 4.

DISCUSSION

Female sexual dysfunction is an overlooked disorder. Many women accept this situation as normal and hesitate to consult a doctor. Additionally, some societies opinion female sexual dysfunction as something to be ashamed of (11). However, a healthy sexual life is essential for a normal life and good partner relationships. We believe that clarifying predictive factors that may be associated with female sexual dysfunction is crucial. Thus, this study was conducted to investigate the correlation between AGD and sexual health for women. Almost one third of women women suffered from sexual dysfunction, and longer AGD_{AC} and GH were associated with female sexual dysfunction and female sexual dissatisfaction.

The AGD_{AC} area includes nerve-rich genitalia clitoris, labia minor, and labia majora. Sertkaya et al. investigated the

Table 3. Demographic data and AGD according to SQOL-F score.

	SQOL-F Score 18-51 (n:77)	**SQOL-F Score 52-84 (n:120)**	**SQOL-F Score >84 (n:83)**	**p value**
Age (years)*	31.3±6.7	30.8±7.4	32.6±7.8	0.281
BMI (kg/m^2)*	26.8±4.8	26.7±4.2	27.6±5.5	0.176
Parity n;(%)				
Nulliparous	16 (20.8)	19 (15.8)	14 (16.9)	
Parity ≥1	61 (79.2)	101 (84.2)	69 (83.1)	0.647
AGD_{AF} (mm)*	24.0±5.3	24.7±6.9	24.6±5.5	0.750
AGD_{AC} (mm)*	77.4±6.2	66.0±9.9	61.5±5.1	***0.001*** **
GH (mm)*	29.5±5.7	22.7±3.7	20.2±4.0	***0.001*** **

*Mean ± standard deviation; AGD_{AC}: Anogenital distance from the anus to the clitoris, AGD_{AF}: Anogenital distance from the anus to the fourchette, BMI: Body mass index, GH: Genital hiatus, SQOL-F: Sexual Quality of Life-Female. **Significant difference according to ANOVA test.

Table 4. Correlation analysis between AGD and sexual function scores.

	FSFI score	SQOL-F Score
AGD_{AF} (mm)		
r	0.025	0.063
p value	0.671	0.294
AGD_{AC} (mm)		
r	-0.546	-0.604
p value	***0.001***	***0.001***
GH (mm)		
r	-0.504	-0.603
p value	***0.001***	***0.001***

AGD_{AC}: Anogenital distance from the anus to the clitoris, AGD_{AF}: Anogenital distance from the anus to the fourchette, GH: Genital hiatus, FSFI: Female Sexual Function Index, SQOL-F: Sexual Quality of Life-Female. (r= 0-0.3 (weak correlation), r= 0.3-0.7 (medium correlation), r= 0.7-1 (strong correlation))

impact of anus-scrotum distance and anus distance on premature ejaculation, and the author found that distances were significantly lower in patients with premature ejaculation (12). In another study, Domenici and colleagues evaluated correlations between AGD and female sexual health and found shorter AGD was related with vulvovaginal atrophy and sexual function impairment. However, Domenici et al. only investigated women in the post-menopausal period (13). In contrast, we found that women with shorter AGD_{AC} had significantly better sexual function. In contrast to Domenici's study, only pre-menopausal women were included in our study. Also, we believe that longer AGD_{AC} may be the result of pelvic trauma, such as vaginal birth, which may be associated with perineal nerve damage and lack of estrogen support. The effect of birth number and hormonal status may be subjects for further investigations. No significant correlation was found between AGD_{AF} and female sexual health.

Previous studies intensively studied the effect of GH on pelvic organ prolapse and incontinence, but not on female sexual function (7, 8). The GH distance is known to directly affect vaginal introitus length and pelvic relaxation may occur due to various factors including aging, vaginal births, obesity, and pelvic surgeries. Many studies reported that many women requested genital aesthetic surgeries due to enlarged vaginal introitus and increased vaginal laxity. Abedi and colleagues compared patients before and after vaginal tightening surgery using FSFI, and the FSFI score of participants increased from 24.19 to 26.92 after vaginal tightening surgery (14). Similarly, Millheiser et al. stated that vaginal tightening with radiofrequency treatment significantly increased female sexual function according to FSFI (15). In the present study, we found that increases in GH were associated with female sexual dysfunction and female sexual dissatisfaction. Due to this outcome, we suggest that female patients with long GH distance should be evaluated for sexual function.

This study has some limitations. This study was done as survey study, and participant answers may have been affected by their current psychological state or the environment. To prevent this situation, all patients answered questions in a silent room without time constraints. Secondly, perineal anatomic features could be affected by race and age; however, we did not focus on these factors, which may be subjects for further studies. Additionally, we could not evaluate duration of sexual dysfunction and AGD at the beginning of sexual dysfunction.

The present study found that almost one third of women had sexual dysfunction. Also, the present study found that longer AGD_{AC} and GH were significantly associated with female sexual dysfunction and female sexual dissatisfaction according to FSFI and SQOL-F for the first time. To better understand the effect of AGD on female sexual health, the present study outcome should be confirmed by further prospective studies with high patient volume. The results of the AGD measurement may be used in the future for the treatment of female sexual function.

Ethics Committee Approval: Ethics committee approval was obtained from the Istanbul Haseki Training and Research Hospital ethics committee (2020-124). All participants signed informed consent.

Author Contributions: Conception/Design of Study- A.G.; Data Analysis- A.G.; Interpretation and Drafting Manuscript- B.H.O.; Critical Revision of Manuscript- A.G.; Final Approval – A.G., B.H.O.

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An *in silico* Investigation of Anticancer Peptide Candidates in Fermented Food Microbiomes

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ABSTRACT

Objective: Cancer is a leading cause of death worldwide, requires development of new effective, specific, and safe strategies that do not carry the disadvantages of traditional cancer treatment approaches. Hence, this study aimed to identify anticancer peptide candidates in fermented food microbiomes through an in *silico* investigation.

Materials and Methods: One hundred eight shotgun metagenomic sequencing samples from six studies on fermented food microbiomes were downloaded from the NCBI and ENA databases and included in the study. Bioinformatic analyses including quality control of raw data, *de novo* assembly, prediction of protein sequences, anticancer peptide predictions by an integrated use of four different prediction tools, toxicity predictions and database comparisons were performed.

Results: One hundred forty-two novel anticancer peptide candidates were identified. Liquor, coffee, kefir fermentation samples contained the greatest numbers of anticancer peptide candidates while sugar, dairy, coconut kefir and brine-type fermentations were dominant sources according to the substrate type.

Conclusion: This study indicates the potential of fermented food microbiomes as a useful source for candidate anticancer peptide detection. *In vitro and in vivo* validations of detected peptides may lead to development of new candidate molecules for cancer therapy in the future.

Keywords: Fermented food, microbiome, bioinformatics, metagenomics, anticancer peptide

INTRODUCTION

Cancer is a leading cause of death worldwide and responsible for an estimated 9.6 million deaths each year (1). According to the Global Cancer Observatory, the most common types of cancer are lung, breast and colorectal cancer (2). Traditional approaches to cancer management, such as radiotherapy, chemotherapy, surgery, can have significant negative effects (3,4). Considering the disadvantages of current treatment approaches such as medical complexities, adverse effects and high treatment costs, the development of effective, specific and safe new strategies is of paramount importance in terms of public health and economic benefits (5).

The focus on peptides as potential anticancer agents gained momentum after studies reporting antimicrobial peptides with varying levels of activity against tumor cells (6). Anticancer peptides show their cytotoxic activities in a similar way to antimicrobial peptides. Many anticancer peptides destroy cancer cells via apoptosis and necrosis by membrane lysis or pore formation (7). Anticancer peptides

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have many advantages such as crossing biological barriers more easily, broad target range, less side effects, less accumulation in tissues and lower toxicity which make them stand out as potential anticancer agents when compared to currently used chemotherapy drugs (8). Peptides with anticancer properties and desirable properties such as specificity, solubility, tumor penetration and safety are among the new alternatives suitable for cancer therapy (9).

Fermented foods have been part of the human diet for several millennia and are consumed in a variety of ways around the world. Fermented foods have been associated with alleviating various health problems in humans (10), and these properties have been attributed to bioactive compounds formed as a result of microbial fermentation (11). In recent years, there has been a growing number of reports on the anticancer effects of fermented foods (12). Anticancer peptides derived from fermented foods have the potential of being used as suitable alternatives to traditional cancer management approaches (5), many computational methods for anti-cancer peptide identification have been developed in the last decade (13). However, considering the massive amount of data produced by high throughput genomics technologies for fermented foods, there is a strong need for efficient strategies for the prediction and testing of anticancer peptide candidates in fermented food microbiomes.

In this study, shotgun metagenomics based fermented food microbiome samples reported by different studies were analyzed to conduct an *in silico* investigation of anticancer peptide candidates encoded within the genomes of the microbiome members using a bioinformatics workflow including metagenome assembly, combined use of anticancer peptide prediction tools, toxicity and novelty analyses.

MATERIALS AND METHODS

One hundred eight shotgun metagenomic sequencing samples from six studies focusing on fermented food microbiomes were downloaded from the National Center for Biotechnology Information (NCBI), and the European Nucleotide Archive (ENA) databases and included in the study. The overall analysis strategy of the study is presented in Figure 1. Quality control of the data was first performed to clean raw sequences. Using trimmomatic, the adapter sequences were removed, the low-quality ends of the sequences were trimmed from the position with the quality score value below 20, and the sequences with a length shorter than 30 bases were eliminated. Quality controlled and filtered clean sequences were used for metagenome assembly analysis which was performed using the default parameters of the metaSPAdes (v3.15.0) (14) and contig sequences were obtained. Protein-coding sequences (CDS) for each of these contigs were predicted and annotated using Prokka (v1.4.0) with option –metagenome (15). Thereafter, CDS with the length between 15 amino acids and 50 amino acids were determined using SeqKit (v2.3.0) (16) and used for downstream analyses. The anticancer activity potentials of these peptides were estimated by four different anticancer peptide prediction tools, AntiCP2 (v2.0) (17), mACPred (v1.0) (18), ACP-MHCNN (v1.0) (19), ACPred (v1.0) (20) using default options for each tool. By combining the results obtained from these tools, the anticancer peptides predicted commonly by all four prediction tools were determined and considered as a reliable set. Next, ToxinPred (v1.0) (21) was used to carry out an *in silico* toxicity analysis. Finally, CancerPPD (v1.0) (22), a manually curated database of experimentally validated anticancer peptides, was used to check the novelty of anticancer peptide, candidates with minimum similarity threshold of 100% and the peptides with the highest anticancer property score and the lowest toxicity were determined.

RESULTS

One hundred eight shotgun metagenomics samples from six studies focusing on fermented food microbiomes were included in this study. Fermented food samples represented five main substrate types, namely brine, coconut kefir, dairy, soy, and sugar (Figure 2). Anticancer peptides candidates were recovered in samples from a total of 13 countries (Cote d'Ivoire, Saudi Arabia, Ecuador, Egypt, Benin, Russia, United Kingdom, Germany, Mexico, China, Ireland, Japan, and Turkiye) across five continents (Asia, Africa, Europe, North America, South America)

Table 1. General characteristics of sequencing data at different steps of bioinformatics analysis.

Study ID	Raw	QC Filtered*	Contigs	CDS*	CDS (15<n<50)	Reference**
PRJEB19968	2,287,242	1,824,414	48,044	36,241	3,348	-
PRJEB21086	26,002,221	22,511,121	86,532	34,884	3,550	-
PRJEB22200	2,275,543,745	1,991,566,237	608,723	308,949	43,830	(29)
PRJEB24129	56,774,402	46,729,715	3,669,361	1,349,589	183,550	(30)
PRJEB35321	347,841,507	263,627,918	7,102,933	2,076,255	305,492	(23)
PRJNA260163	1,683,868,773	1,425,472,508	12,142,357	7,586,687	444,668	-
Total	4,392,317,890	3,751,731,913	23,657,950	11,392,605	984,438	

*QC: Quality Control Filtered, CDS: Protein Coding Sequence, **3 studies did not have any associated publication in the NCBI and ENA databases

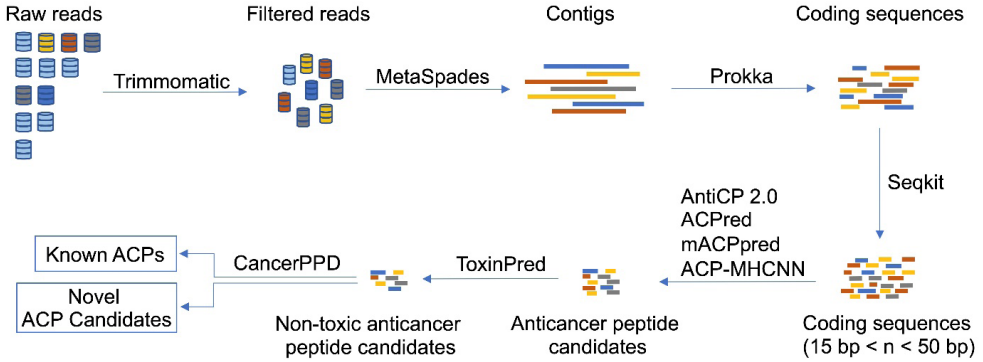

Figure 1. Overall analysis strategy.

(Figure 2). The majority of samples were collected in China (n=24), Ireland (n=24) and Turkiye (12).

To predict anticancer peptide candidates in fermented food microbiomes, a total of 4.4 billion reads were analyzed (Table 1). Metagenome assemblies yielded 23.6 million contigs. Then, CDS for each of these contigs were predicted and a combined set of 11.4 million CDS were obtained among which 984,438 short CDS (between 15 amino acids and 50 amino acids in length) were used for prediction of anticancer peptide candidates.

Anticancer peptide predictions were performed by combining four different anticancer peptide prediction tools, namely AntiCP2, mACPred, ACP-MHCNN and ACPred. ACPred predicted the highest number of anticancer peptide candidates (n=80,646) while AntiCP2 predicted the lowest number of anticancer peptide candidates (n=7,570; Figure 3). Since the correct identification of anticancer peptide candidates vary between different methods, only 168 anticancer peptide candidates predicted by all tools were considered as reliable and used for downstream analyses. The overlap between all four prediction tools was approximately 0.001% (168 anticancer peptide candidates in 133,263 anticancer peptide candidates predicted in total).

One hundred sixty-eight anticancer peptide candidates commonly predicted by all prediction tools were further analyzed to assess their anticancer activity potential. These anticancer peptides were first tracked back to the studies and samples they originated from. The results showed liquor, coffee, and kefir fermentation samples as main sources of these anticancer peptides while sugar and brine type fermentations were main substrate types (Figure 4). Next, the potential toxicity and novelty of 168 anticancer peptide candidates were examined which resulted in 142 anticancer peptide candidates predicted as non-toxin and have not been reported to date (Supplementary File 1).

DISCUSSION

Microorganisms involved in the fermentation process play an important role in the formation of health associated properties of fermented foods (23). It is known that the bioactive molecules produced by these microorganisms could have anti-inflammatory, antifungal, antibiotic, or anticancer properties (12). Thus, examination of microbial genomes can lead to the discovery of new biological agents used in treatment of a variety of diseases, or facilitating biotechnological process. Considering the increasing number of reports on the anticancer effects of fermented food (12), the genomes of microorganisms participating in the fermentation process may be a potential reservoir for novel anticancer peptides. Thanks to the growing

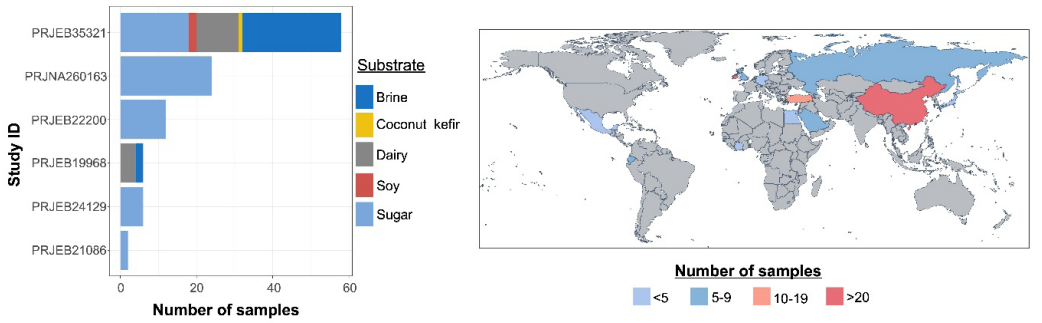

Figure 2. Number of samples for each study used to predict anticancer peptide candidates, colored according to fermented-food substrates. Geographic distribution of the number of samples retrieved per country.

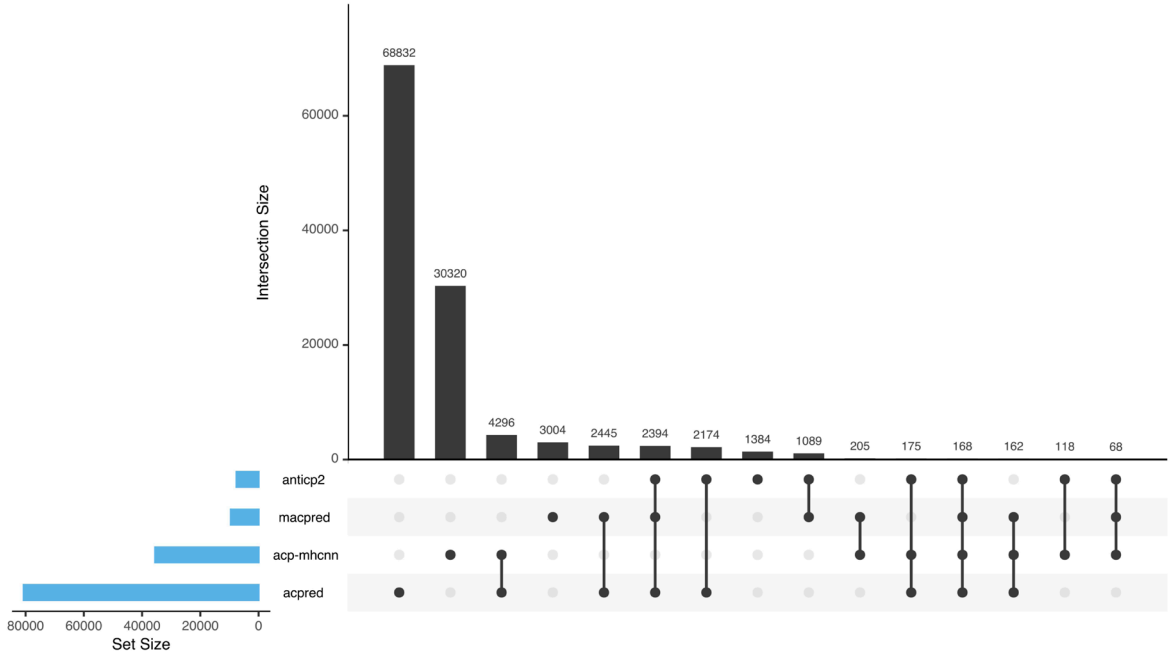

Figure 3. Number of predicted anticancer peptide candidates across anticancer peptide prediction tools. Vertical bars represent the number of predicted anticancer peptide candidates shared between the specific tools highlighted with connected dots in the lower panel. Horizontal bars in the lower panel indicate the total number of anticancer peptide candidates predicted by each prediction tool.

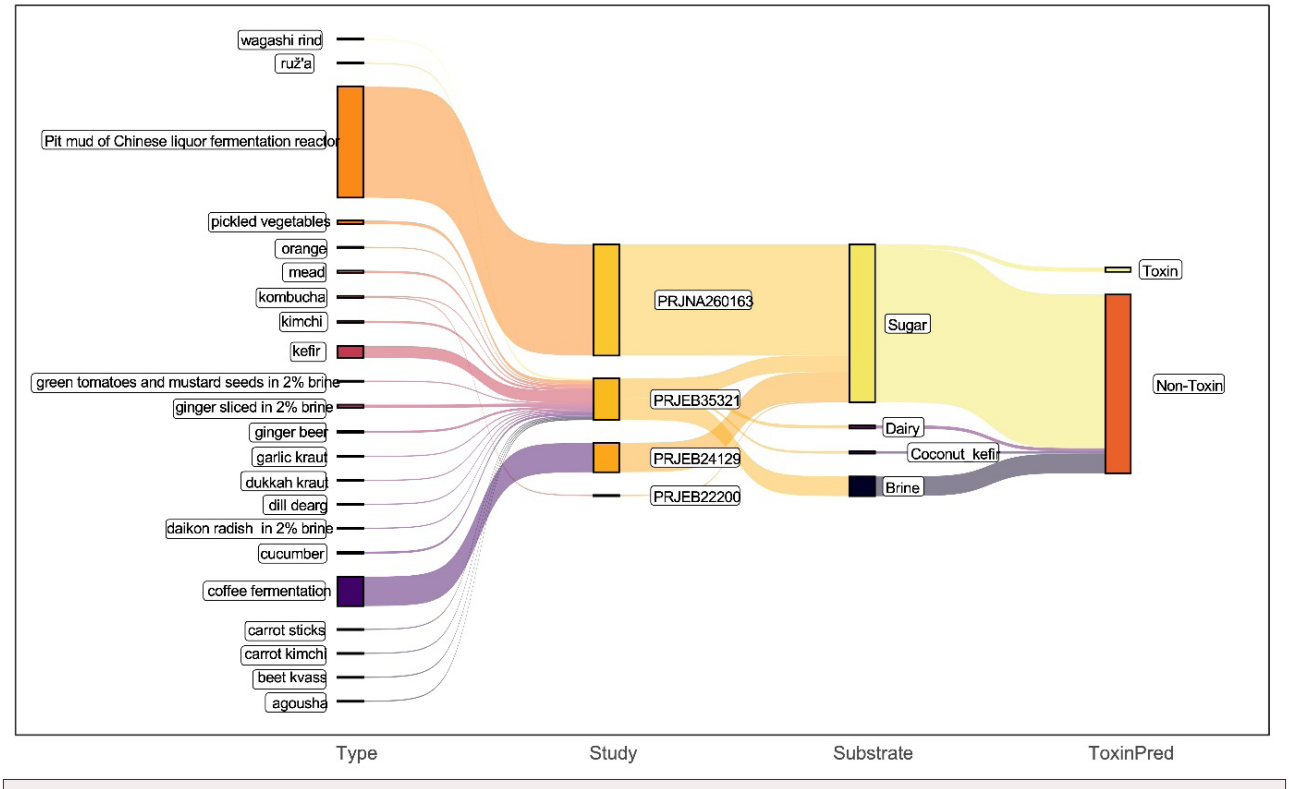

Figure 4. Sankey plot displaying distribution of 168 anticancer peptide candidates across fermented food, study, substrate type and predicted toxicity categories.

use of the next generation sequencing technologies and development of bioinformatics tools, the microbial genomes can be screened for anticancer peptide candidates through much more comprehensive approaches (13).

In this study, microbiomes of many fermented food types originating from different countries were analyzed together to detect anticancer peptide candidates. Starting with billions of DNA sequencing reads, 142 novel anticancer peptide candidates have been predicted and reported. One of the findings of the study was the high-level inconsistency between different anticancer peptide prediction tools which suggests that caution is needed when using a single anticancer peptide prediction tool. Thus, a consensus approach based on multiple anticancer peptide prediction tools -as applied in this study- can be used to alleviate this issue and ensure robust predictions. Moreover, the size distribution after length-based filtering of CDS yielded peptide sequences with lengths ranging from 30 amino acids to 50 amino acids and potentially caused missing shorter anticancer peptide candidates. As this is a known limitation related to software used for CDS prediction and annotations, new tools for small open reading frames would serve as valuable tools to overcome this limitation in the future (24).

The detected anticancer peptide candidates mainly originated from liquor, coffee and kefir fermentations which are classified as sugar, dairy and brine type fermentations. Among these fermented foods, interestingly, kefir has been reported to have anticancer effects by several studies (25–28). This distribution could be partly attributed to the number of raw reads obtained from the samples; however, interestingly, there was very low number of anticancer peptide candidates detected in other samples sequenced with very high coverage such as kombucha samples. It should also be noted that the microbial diversity in the samples potentially have significant effects on the number of the predicted anticancer peptides. The toxicity analysis and comparison with the previously characterized anticancer peptides revealed that most of the predicted anticancer peptide candidates have not been reported before. Considering the high throughput characteristics of the approach applied in this study, comprehensive high throughput wet laboratory testing, and characterization methods will be strongly needed in the future.

Fermented food microbiomes were found to be a useful source for candidate anticancer peptide discovery. *In vitro* and *in vivo* validations of the identified peptides may lead to development of new candidate molecules that can be used in cancer therapy.

Ethics Committee Approval: Ethics committee approval is not required because of no material or experimental animal that would require permission.

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SUPPLEMENTARY INFORMATION

Supplementary File 1. The sequences of 142 novel anticancer peptide candidates determined in the study.

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>acp2
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3 Final approval of the version to be published; AND
4 Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Manuscript Types

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Table 1. Limitations for each manuscript type

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Original Article	3500	200 (Structured)	30	6	7 or total of 15 images
Review Article	5000	200	50	6	10 or total of 20 images
Case Report	1000	200	15	No tables	10 or total of 20 images
Letter to the Editor	500	No abstract	5	No tables	No media

dimensions: 100 × 100 mm). Figure legends should be listed at the end of the main document.

All acronyms and abbreviations used in the manuscript should be defined at first use, both in the abstract and in the main text. The abbreviation should be provided in parentheses following the definition.

When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

All references, tables, and figures should be referred to within the main text, and they should be numbered consecutively in the order they are referred to within the main text.

Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

References

While citing publications, preference should be given to the latest, most up-to-date publications. Authors are responsible for the accuracy of references. References should be prepared according to Vancouver reference style. If an ahead-of-print publication is cited, the DOI number should be provided. Journal titles should be abbreviated in accordance with the journal abbreviations in Index Medicus/ MEDLINE/PubMed. When there are six or fewer authors, all authors should be listed. If there are seven or more authors, the first six authors should be listed followed by "et al." In the main text of the manuscript, references should be cited using Arabic numbers in parentheses. The reference styles for different types of publications are presented in the following examples.

Journal Article: Rankovic A, Rancic N, Jovanovic M, Ivanović M, Gajović O, Lazić Z, et al. Impact of imaging diagnostics on the budget – Are we spending too much? Vojnosanit Pregl 2013; 70: 709-11.

Book Section: Suh KN, Keystone JS. Malaria and babesiosis. Gorbach SL, Barlett JG, Blacklow NR, editors. Infectious Diseases. Philadelphia: Lippincott Williams; 2004.p.2290-308.

Books with a Single Author: Sweetman SC. Martindale the Complete Drug Reference. 34th ed. London: Pharmaceutical Press; 2005.

Editor(s) as Author: Huizing EH, de Groot JAM, editors. Functional reconstructive nasal surgery. Stuttgart-New York: Thieme; 2003.

Conference Proceedings: Bengisson S. Sothemin BG. Enforcement of data protection, privacy and security in medical informatics. In: Lun KC, Degoulet P, Piemme TE, Rienhoff O, editors. MEDINFO 92. Proceedings of the 7th World Congress on Medical Informatics; 1992 Sept 6-10; Geneva, Switzerland. Amsterdam: North-Holland; 1992. pp.1561-5.

Scientific or Technical Report: Cusick M, Chew EY, Hoogwerf B, Agrón E, Wu L, Lindley A, et al. Early Treatment Diabetic Retinopathy Study Research Group. Risk factors for renal replacement therapy in the Early Treatment Diabetic Retinopathy Study (ETDRS), Early Treatment Diabetic Retinopathy Study Kidney Int: 2004. Report No: 26.

Thesis: Yılmaz B. Ankara Üniversitesindeki Öğrencilerin Beslenme Durumları, Fiziksel Aktiviteleri ve Beden Kitle İndeksleri Kan Lipidleri Arasındaki Ilişkiler. H.Ü. Sağlık Bilimleri Enstitüsü, Doktora Tezi. 2007.

Manuscripts Accepted for Publication, Not Published Yet: Slots J. The microflora of black stain on human primary teeth. Scand J Dent Res. 1974.

Epub Ahead of Print Articles: Cai L, Yeh BM, Westphalen AC, Roberts JP, Wang ZJ. Adult living donor liver imaging. Diagn Interv Radiol. 2016 Feb 24. doi: 10.5152/dir.2016.15323. [Epub ahead of print].

Manuscripts Published in Electronic Format: Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis (serial online) 1995 Jan-Mar (cited 1996 June 5): 1(1): (24 screens). Available from: URL: http:/ www.cdc.gov/ncidodlEID/cid.htm.

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