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Evaluation of miR-146a Expression Levels in Archived Serum Samples for the Diagnosis/Follow-up of Patients with Cystic Echinococcosis

Kistik Ekinokokkozisli Hastaların Tanısı/Takibinde Kullanılan Arşiv Serum Örneklerinde miR-146a Ekspresyon Düzeylerinin Değerlendirilmesi

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ABSTRACT

Objective: Cystic echinococcosis (CE) is a zoonotic disease that causes fluid-filled cysts in internal organs and is a major public health problem worldwide. The lack of standardized methods for the diagnosis/follow-up of CE disease necessitates the development of new non-invasive diagnostic tools, such as the determination of changes in the expression levels of circulating microRNAs (miRNA). In this study, we aimed to investigate the presence of miR-146a in archived serum samples of CE patients for the first time and to evaluate its potential role in the diagnosis and follow-up of CE over a three-year period.
Methods: This study included archived serum samples from 39 CE patients, 56 follow-up samples from 14 CE patients, and 3 healthy controls, and expression levels of miR-146a were evaluated in each group using quantitative real-time polymerase chain reaction. Due to the small and unbalanced control group, bootstrapped confidence intervals were used; time-dependent changes in follow-up patients were analyzed using a linear mixed-effects model and Welch's F test to address variance heterogeneity.
Results: The *miR146* gene was found to be significantly upregulated in archived serum samples of patients with CE compared to healthy control samples. Additionally, the expression level of the *miR146* gene in follow-up serum samples significantly decreased in the third year post-surgery compared to follow-up blood samples taken in previous years ($p<0.05$).
Conclusion: According to the obtained results, it was concluded that miR-146a can be recommended as a diagnostic biomarker in the diagnosis and follow-up of CE, and archived materials of CE patients can be utilized in new biomarker research.
Keywords: Cystic echinococcosis, MicroRNA, diagnosis, patient follow-up, archive serum

ÖZ

Amaç: Kistik ekinokokkozis (KE), iç organlarda sıvı dolu kistlere neden olan zoonotik bir hastalıktır ve dünya çapında önemli bir halk sağlığı sorunudur. KE hastalığının tanısı/takibi için standardize edilmiş yöntemlerin eksikliği, dolaşımdaki mikroRNA'ların (miRNA'lar) ifade seviyelerindeki değişikliklerin belirlenmesi gibi yeni invaziv olmayan tanı araçlarının geliştirilmesini gerekli kılmaktadır. Bu çalışmada, ilk kez KE hastalarının arşiv serum örneklerinde miR-146a'nın varlığını araştırmayı ve üç yıllık bir süre boyunca KE'nin tanısı ve takibindeki potansiyel rolünü değerlendirmeyi amaçladık.
Yöntemler: Bu çalışmaya 39 CE hastasının arşiv serum örneği, 14 CE hastasının 56 takip örneği ve sağlıklı kontrollerden 3 örnek dahil edildi ve miR-146a'nın ifade düzeyleri her grupta kantitatif gerçek-zamanlı polimeraz zincir reaksiyonu kullanılarak değerlendirildi. Grup karşılaştırmaları için küçük ve dengesiz kontrol grubuna bağlı olarak yeniden örneklemeli güven aralıkları kullanıldı; takip hastalarındaki zamana bağlı değişimler ise varyans heterojenliğini dikkate alan lineer karma etkili model ve Welch F testi ile analiz edildi.
Bulgular: *miR146* geninin, CE'li hastaların arşivlenmiş serum örneklerinde sağlıklı kontrol örneklerine kıyasla ifade düzeyinin önemli ölçüde arttığı bulunmuştur. Ayrıca, takip serum örneklerindeki *miR146* geninin ifade düzeyi, ameliyattan sonraki üçüncü yılda önceki yıllarda alınan takip kan örneklerine kıyasla önemli ölçüde azalmıştır ($p<0,05$).
Sonuç: Çalışmamızın sonuçlara göre miR-146a'nın KE'nin tanı ve takibinde tanısal biyobelirteç olarak önerilebileceği ve KE hastalarına ait arşiv materyallerinin yeni biyobelirteç araştırmalarında kullanılabileceği sonucuna varılmıştır.
Anahtar Kelimeler: Kistik ekinokokkozis, MikroRNA, tanı, hasta takibi, arşiv serum

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Cystic echinococcosis (CE) is a zoonotic disease caused by the metacestode form of *Echinococcus granulosus*, characterized by the development of fluid-filled cysts in internal organs. CE is a public health issue affecting people from all age groups and is endemic in many countries, including Türkiye (1,2).

In the treatment of CE, the choice of method including surgery, PAIR (puncture, aspiration, injection, reaspiration), medical therapy, or "watch and wait" depends on the characteristics of the cyst, such as its location and stage. Following the treatment of CE, recurrence may reach up to 25%, making the management of the disease difficult (3).

The diagnosis of CE primarily relies on clinical findings and imaging techniques, but combining imaging and serological tests is recommended due to complicated early diagnosis (3). However, the accuracy of serological tests can be influenced by factors related to the patient, such as the stage, location, number, and size of the cyst, and seropositivity varies between 56% and 100% in different laboratories. Serologic tests have also limited performance in the follow-up of CE due to the long-term presence of disease-specific antibodies, the possibility of false results in seronegative patients, and the inability to distinguish between inactive and recurrent cysts (4).

The lack of standard methods for the diagnosis/follow-up of disease reveals the need for the development of new diagnostic tools such as liquid biopsy. Circulating free microRNAs (miRNAs) serve as biomarkers in various fields, including oncology, neurology, cardiology, spinal surgery, epilepsy, and neurodegenerative diseases, and play a critical role in modulating gene expression and many biological processes (5). miRNAs exhibit stability in body fluids such as serum and plasma due to their resistance to ribonuclease (RNase) activity, high temperatures, repeated freeze-thaw cycles, extreme pH levels, and long-term storage, allowing the analysis of their expression levels even in archived materials (6).

Parasitic infections affect both parasite and host miRNAs, leading to changes in host immune responses. This suggests that monitoring alterations in parasite-derived and/or parasite-specific miRNAs may help identify diagnostic biomarkers in parasitology (7-11).

In the present research, we have aimed to investigate the presence and changes in expression levels of miR-146a in serum samples collected during a three-year follow-up of CE patients; to our knowledge, this is the first study to assess its expression in archived serum samples.

METHODS

Ethics Statement

The present research received approval from Ethics Committee of Ege University Faculty of Medicine (approval no: 2023-0780/23-4.1T/67) and consent forms were obtained from the participants.

Clinical Samples

The study included archived serum samples of 39 patients (first blood samples with the letter "a" code taken during surgery) who were admitted to Ege University Hospitals between 2016 and 2019 and diagnosed with CE through radiological and serological examinations, and 3 healthy controls without underlying chronic diseases.

In addition, a total of 56 follow-up serum samples collected from 14 CE patients on the day of surgery (day 0), as well as one year, two years, and three years after surgery, were included [coded as the first (a), second (b), third (c) and fourth (d) blood samples for each follow-up CE patient, respectively]. During the three-year follow-up period, no recurrence of the disease was detected by serological and imaging methods. All materials were stored at -20 °C.

RNA Isolation

Total RNA was isolated using the mirVana™ miRNA Isolation Kit (Invitrogen) using 200 µL of archived serum samples from CE patients and control groups. Concentration and purity were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNAs were stored at -80 °C until cDNA synthesis.

TaqMan miRNA Assay

Complementary DNAs (cDNAs) were synthesized from total RNA, including miRNA, using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time polymerase chain reaction was performed using the TaqMan® MicroRNA Analysis Kit to measure miRNA expression, and the primers used for miR-146a-5p and endogenous miR-16-5p are listed in Table 1. In this study, hsa-miR-16-5p was used as an endogenous miRNA for normalization in humans due to its suitability for miRNA studies reported in the literature (12,13). We calculated the relative expression levels of miRNAs using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

In the analysis of *miR-146* gene expression between control and archived first blood samples, the dataset was highly unbalanced, with only three observations in the healthy control group. Given the small sample size in the healthy control group, we determined that standard parametric and non-parametric tests were not appropriate. Instead, we used bootstrapped confidence intervals (CIs) to estimate the median response in each group, utilizing the boot package in R (14,15). We generated a total of 1,000 bootstrap resamples to construct 95% CIs for the group medians. Statistical significance was assessed by examining the overlap of CIs, where non-overlapping CIs suggest a potential difference between groups.

To analyze the effect of time on the individuals being followed up, a random intercept general linear model was created by optimizing the log-likelihood function, utilizing the lme4 and lmertest libraries (16,17). The model was formulated as follows: lmer(response ~ time + (1 | patient_id), data = data, REML = FALSE).

The intraclass correlation coefficient (ICC=0.064) indicated that only 6.4% of the total variance was due to differences between individuals, suggesting a weak dependency within subjects. The likelihood ratio test (LRT=0.19, p=0.66) confirmed that including a random intercept did not significantly improve the model. Given that the random intercept was not significant, we proceeded with a One-Way ANOVA framework to analyze time effects. Initially, we tested the data for normality and heterogeneity by using the Shapiro-Wilk test, Q_Q plots, and Levene's test. As there

Table 1. The TaqMan probes and miR sequences

miRBase ID	Assay ID	Mature miRNA sequence
hsa-miR-146a-5p	000468	UGAGAACUGAAUUCCAUGGGUU
hsa-miR-16	000391	UAGCAGCACGUAAAUAUUGGCG

Table 2. Demographic and clinical characteristics of 39 patients with CE

Patient	Gender	Age (yrs.)	Cyst number	Cyst location	Cyst size	Cyst stage
1	Male	64	1	Liver	M	CE3B
2	Male	42	Multiple	Liver	M	CE4
3*	Female	16	Multiple	Liver	M	CE1
4*	Female	44	1	Liver	L	CE2
5	Female	23	1	Liver	M	CE1
6	Male	26	Multiple	Liver	M	CE2
7	Female	63	Multiple	Liver	M	CE2
8*	Female	10	1	Lung	M	CE1
9*	Male	9	Multiple	Liver	M	CE1
10	Female	73	1	Liver	M	CE2
11	Female	18	1	Liver	L	CE1
12*	Male	25	Multiple	Liver	M	CE1
13*	Male	56	Multiple	Liver	L	CE3B
14*	Female	9	1	Liver	M	CE2
15	Male	62	Multiple	Liver	M	CE1
16	Male	42	Multiple	Liver	L	CE1
17	Female	50	Multiple	Liver	M	CE1
18*	Female	25	1	Liver	M	CE1
19*	Female	5	1	Liver	S	CE2
20*	Female	38	Multiple	Liver	L	CE1
21*	Male	13	Multiple	Liver	L	CE1
22*	Male	14	1	Lung	M	CE1
23	Male	62	Multiple	Liver	M	CE1
24*	Female	58	1	Liver	L	CE1
25	Female	30	Multiple	Liver	L	CE1
26*	Female	11	Multiple	Lung	L	CE3A
27	Male	5	Multiple	Lung	M	CE1
28	Female	85	1	Liver	L	CE2
29	Male	63	1	Liver	L	CE2
30	Female	13	Multiple	Liver	S	CE1
31	Male	7	Multiple	Liver	M	CE1
32	Female	9	1	Lung	L	CE2
33	Male	4	1	Liver	L	CE1
34	Female	59	1	Liver	L	CE1
35	Male	65	Multiple	Liver	L	CE4
36	Female	10	1	Lung	M	CE3A
37	Female	9	Multiple	Liver	S	CE3B
38	Female	26	1	Liver	L	CE2
39	Male	10	1	Liver	M	CE1

*: Follow-up patients, S: Small (≤5), M: Medium (5<-<10), L: Large (10≤), CE: Cystic echinococcosis

were issues with normality and heterogeneity, a square root transformation resolved the normality problem, but heterogeneity remained an issue. Therefore, we applied Welch's heteroscedastic F test which provides a robust alternative to standard ANOVA under variance heterogeneity. For post-hoc comparisons, we conducted pairwise Welch t-tests with Bonferroni correction using the R library onewaytest (18).

GraphPad Prism 8.0 (GraphPad software, CA) was used for other data analyses. Receiver operating characteristic (ROC) curve, Mann-Whitney U tests, unpaired t-tests, and One-Way ANOVA were also used for the relevant data analyses. Outliers were identified using the ROUT method with a Q value of 1%.

RESULTS

Characteristics of Patients

Among the 39 patients with CE, ages ranged from 4 to 85 years, with 41% under 18. The majority were female (56.4%), and all cysts showed liver localization except six cysts in the lungs. According to the World Health Organization, 95% of the cysts were in the active (CE1 and CE2) and transitional stages (CE3A and CE3B). Detailed demographic and clinical data are in Table 2.

miR146 Gene Expression Analyses

In this study, it was shown that the *miR146* gene was significantly up-regulated in the archived first blood samples of patients with CE compared to archived samples from a healthy control group. Figure 1 showed that miR-146a expression was elevated in archived serum samples from CE patients compared to healthy controls. In addition, the differences found between these two groups were detected to be statistically significant ($p<0.05$). The fold-change of *miR146* in CE patients was calculated to be 21.05. Furthermore, the area under the ROC for these groups

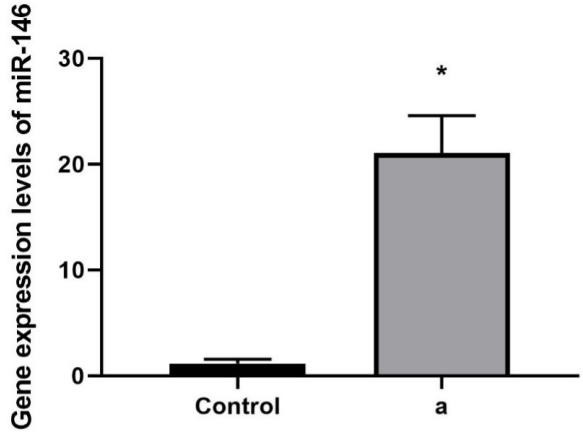

Figure 1. Gene expression levels of miR-146. The control group consists of archived samples from healthy individuals. Group "a" displays archived first blood samples from patients with CE. An asterisk ("*") denotes a significant difference between the CE patient group and the healthy control group ($p<0.05$). The data are expressed as the mean ± SEM

CE: Cystic echinococcosis, SEM: Standard error of the mean

was 0.9355, with a p-value of 0.0139. Figure 2 demonstrated the ROC analysis of *miR-146a*, showing its ability to distinguish CE patients from healthy control.

When examining the expression levels of the *miR146* gene in follow-up patients over the years, it was found that miR146 expression decreased in the third blood sample (c) compared to the first (a) and second (b) samples. However, these found differences were not statistically significant. In contrast, the fourth blood sample (d), collected in the third year, demonstrated a significant decrease in miR146 expression compared to the samples collected in the previous years. Figure 3 showed *miR-146a* gene expression levels in CE patients during three-year follow-up.

The expression level of miR-146 was analyzed in categorized CE patients, showing no significant differences with respect to gender, age, cyst number, cyst stage, or cyst localization.

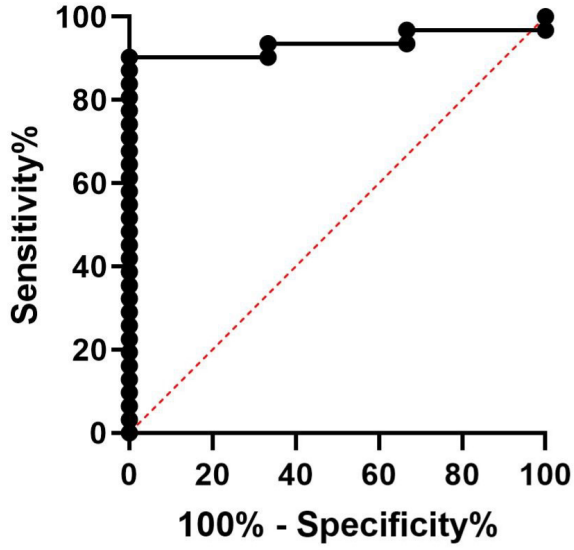

Figure 2. Receiving operating characteristic analysis of *miR146* gene expression level between healthy controls and the disease group

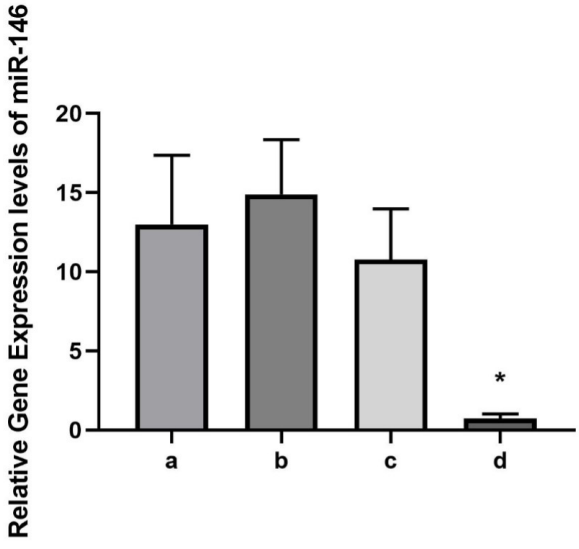

Figure 3. Gene expression levels of miR-146 in follow-up patients over the years. a (day 0), b (year 1), c (year 2), and d (year 3). The asterisk (*) indicates a significant difference found between the d group and all other follow-up groups ($p<0.05$). Data are shown as the mean ± SEM

SEM: Standard error of the mean

DISCUSSION

MicroRNAs (miRNAs), which consist of 17 to 25 nucleotides, are single-stranded, non-coding RNA that significantly contribute to host-pathogen interactions as well as the host's immune responses to microbial agents (19). During parasitic infection, microRNAs expressed in the host, either derived from parasites or specific to parasites, are passively or actively circulating in exosomes, and alterations in the expression levels of these miRNAs could provide a new approach in diagnostic and prognostic biomarker research (5).

In previous studies, miRNA pathway analysis was investigated at different life cycle stages of *Echinococcus* species. Notably, *E. granulosus* (egr)-miR-125-5p and egr-miR-2a-3p were shown to be abundant in protoscoleces (20). A mouse model study showed that pro-inflammatory factors like tumor necrosis factor (TNF) α, interleukin (IL)12, and IL6 inhibit key signaling pathways in CE (21). Additionally, Ren et al. (22) reported that miR-483-3p found in liver tissues and plasma could be suggested as a potential diagnostic biomarker for alveolar echinococcosis (AE).

This study investigated the expression levels of miRNA-146a in archived serum samples of patients with CE. This study is, to the best of our knowledge, the first investigation of the expression of particular miRNAs in archived sera from patients with CE. Recent research has explored how long-term storage conditions affect the stabilization of miRNAs in archived materials and their potential use in developing biomarkers. Rounge and colleagues investigated the quantity and quality of miRNA in serum samples from one of the world's oldest biobanks, stored at -20 °C for a duration of up to 40 years (6). Their findings confirm that the targeted miRNAs remained stable in archived serum materials after long-term storage, consistent with results from other studies with similar goals (23,24).

miR-146a plays an important role in regulating the Th1 response during helminth infections by reducing the release of IFN-γ and TNF-α. It also triggers and modulates the Th2 response in both allergic reactions and helminth infections (10,25). Mahami-Oskouei et al. (10) demonstrated in their study, which included 20 CE patients and 20 healthy controls, that miR-146a was significantly up-regulated in CE patient plasma samples. Similarly, Eroglu et al. (9) showed that miRNA 146a levels were higher in tissue samples from 30 CE patients and 20 AE patients compared to healthy individuals. In parallel with these studies, our research indicated that *miR-146* gene expression was notably elevated in archived blood samples from CE patients taken at the time of surgery compared to archived samples from healthy controls. Furthermore, ROC analysis indicates that the increase in miR-146 can be used as a biomarker for diagnosing CE.

Since the recurrence rate of CE can reach up to 25%, it is necessary to follow-up the patients after treatment. Current diagnostic methods, including imaging and serological tests, are inadequate for early diagnosis of CE and its recurrences. Zhang et al. (26) suggested that protoscoleces shed from cysts may lead to higher

expression levels in patients with active CE cysts. Monitoring changes in miRNA expression during recurrence could provide a new diagnostic and prognostic biomarker approach (26).

Alizadeh et al. (7) showed that egr- miR-71 and egr-let-7 expression levels in the plasma of patients with CE were significantly reduced three and six months after surgery and suggested that miRNAs derived from parasites (egr-miR-71) may be a promising diagnostic biomarker for early CE diagnosis and follow-up. Another study found that the levels of serum egr-miR-2a-3p in patients with CE were much higher than in the control groups, but these levels dropped significantly six months after surgery compared to the initial values (27).

In our study, it was observed that the expression level of the *miR146* gene in the blood samples of the patients followed up significantly decreased in the samples taken in the third year post-surgery, compared to the follow-up blood samples taken in previous years. Additionally, the fact that the same patient group showed no recurrence after three years of follow-up, as determined by serological and imaging methods, suggests that miR-146 could be an important biomarker candidate for follow-up CE patients by tracking changes in expression levels.

Another limitation in the diagnosis of CE is that serological tests cannot differentiate between active and inactive cysts, which complicates the monitoring of the disease. Th2 immune responses are crucial for protective immunity in active cysts, while Th1 responses are associated with immunity in inactive cysts (26,28). In a study aimed at defining the miRNA profile and potential cellular pathways in patients with CE, it was found that several miRNAs—specifically hsa-miR-4692, hsa-miR-181b-3p, hsa-miR-4491, hsa-miR-4518, hsa-miR-4659a-5p, and hsa-miR-3977— were downregulated, likely due to the presence of CE cysts. These miRNAs are associated with cellular processes such as cellular proliferation and apoptosis and they could potentially play a role in anti-cancer effects (11). Mariconti et al. (29) revealed that six immune-related miRNAs (miR- 223-3p, let-7g-5p, miR- 16-5p, let-7a-5p, miR- 26a-5p, miR- 30c-5p, miR- 195-5p and miR- 26b-5p) were up-regulated in CE patients with active cyst compared to CE patients with inactive cyst, indicating host-parasite interaction. Örsten et al. (13) focused on investigating the miRNAs derived from parasites in the serum samples of CE patients with active and inactive cysts and healthy controls; they reported that egr egr-miR71-5p, let-7-5p, and egr-miR-9-5p were more expressed in CE patients with active cysts than in those with inactive cysts. Since our study consisted of patients who underwent surgery and therefore mostly had active and transitional cysts, alteration in mir146a expression levels between active and inactive cyst stages may not have been thoroughly evaluated, but no statistically significant difference was observed in miR146a expression levels considering the cyst stages.

Considering the compatibility of the results of our study with similar CE-miRNA studies mentioned above, it is thought that miRNAs could be used as potential diagnostic biomarkers for the diagnosis/follow-up of CE.

Study Limitations

There are several limitations in our study due to the use of archived serum samples from CE patients with active cysts, which were used in a previous serological study. One of these is that the expression levels of target miRNAs could not be investigated in this patient group and could not be compared with CE patients with active cysts due to the small number of patients in the inactive cyst stage in our study. Additionally, the use of archived serum samples limited access to a healthy group. The potential of miR146a in CE patient diagnosis/follow- up needs to be validated with a larger sample group including all clinical variables.

Conclusions

Although there is an increase in studies on the use of circulating miRNAs in the diagnosis/follow-up of CE patients, the presence of microRNAs was demonstrated for the first time in archived serum samples of CE patients used in serological tests and stored at -20 degrees, providing a starting point for the search for new diagnostic biomarkers in CE archived materials. In addition, as a result of demonstrating the presence of miR-146a in archive serum samples and examining the alterations in expression levels during the three-year follow-up period, it was concluded that miR-146a could be used as a candidate diagnostic biomarker in the diagnosis and follow-up of CE.

*Ethics

Ethics Committee Approval: The present research received approval from Ethics Committee of Ege University Faculty of Medicine (approval no: 2023-0780 /23-4.1T/67).

Informed Consent: Consent forms were obtained from the participants.

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Footnotes

*Authorship Contributions

Concept: E.A.Ö., M.A., Ç.C., A.Ü., Design: E.A.Ö., M.A., A.Ü., Data Collection or Processing: E.A.Ö., M.A., Ç.C., A.Ü., Analysis or Interpretation: E.A.Ö., Ç.C., Literature Search: E.A.Ö., M.A., Ç.C., A.Ü., Writing: E.A.Ö., M.A., Ç.C.

Conflict of Interest: No conflict of interest was declared by the authors.

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