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Fibroblast growth factor receptor 1 gene (FGFR1) expression in serous ovarian tumors

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Abstract

Aim: The aim of this study was to evaluate the ovarian serous surface epithelial tumors in terms of FGFR1 expression and to evaluate the differences in expression of serous cystadenomas, borderline serous tumor/atypical proliferative serous tumors, low-grade and high-grade serous carcinomas.

Materials and Methods: A hundred patients diagnosed with serous cystadenomas, borderline serous tumor/atypical proliferative serous tumors (BST), low-grade (LGSOC) and high-grade serous ovarian carcinomas (HGSOC) between 2010-2020 in our pathology laboratory were included in the study. In these cases, real-time PCR and immunohistochemistry were performed on sections from paraffin blocks.

Results: In our study, it was determined that FGFR1 gene expression was statistically significantly increased in HGSOC cases compared to the BST/ LGSOC group and the SC group. Although there was a difference between the BST/ LGSOC group and the SC group, no statistical significance was found.

Conclusion: FGFR1 expression was significantly increased in HGSOC cases. This finding has led to the conclusion that angiogenesis inhibition by FGFR inhibition may be a treatment option in HGSOC cases.

Introduction

Although the incidence of ovarian cancer is low compared to other tumors, it draws attention with its high mortality. Due to the nonspecific symptoms in the early stage, it is diagnosed in the advanced stages and its prognosis is relatively poor. Age at the time of diagnosis, FIGO stage, and tumor type are factors that affect prognosis [1]. Approximately half of the ovarian tumors are epithelial tumors. Studies on the pathogenesis of serous malignant ovarian tumors show that this group consists of 2 separate carcinoma groups, low-grade and high-grade serous carcinoma. Low-grade serous carcinoma (LGSOC) as a type 1 prototype contains a high rate of KRAS and BRAF mutations, but no TP53 mutations. High-grade serous carcinoma (HGSOC) as its type 2 prototype is characterized by high-grade genetic instability and the TP53 mutation found in almost all cases. The formation of these 2 groups of tumors occurs by separate mechanisms. Borderline serous tumor/atypical proliferative tumor (BST) is the precursor of LGSOC [1, 2]. Surgery is the first-choice treatment for tumor burden reduction and staging. Platinum-based chemotherapy is also widely used. Hormonotherapy and targeted therapy can be applied in addition. Expression differences, which can be prognostic markers in the treatment of all tumors and guide new treatments, are being studied intensively [1].

FGF (Fibroblast Growth Factor) pathway is also at an important point in terms of being an angiogenesis stimulator. Many treatments targeting this pathway are in drug trials. FGF can be targeted and its receptors targeted as a treatment option. Differences in expression rates are important when determining the tumors in which these treatments under development can be used. FGF receptors (FGFR) are encoded by 4 genes. These receptors have tyrosine kinase activity. In addition to their receptor functions for fibroblast growth factors, they are involved in embryonal development, cell proliferation, differentiation and migration. With this information, it was thought that an increase in FGF or an increase in FGFR activation might cause tumor growth. In studies conducted in this direction, it has been determined that FGFR gene aberrations are increased in tumor cells and the most common of these aberrations is FGFR1 amplification [3]. It has been determined that the amplification, that is, the pathological activation of FGFR1, has a role in ovarian cancers,

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breast cancers, oral and esophageal squamous cell cancers, lung and prostate cancers. It has been reported that this amplification is detected in approximately 5% of ovarian cancers. However, studies on subgroups of ovarian tumors are limited. In this study, it was aimed to obtain data on the usability of new targets in the treatment of serous ovarian tumors and to create a database for future studies [4, 5].

Materials and Methods

Patient cohort

Cases diagnosed as serous ovarian tumor with morphological and immunohistochemical findings between 2010-2020 in the laboratory of Inonu University Faculty of Medicine, Department of Medical Pathology were scanned in the archive. The control group (serous cystadenoma) was kept in an acceptable number and other serous tumors were accepted to the study as the budget allowed. Cases diagnosed with serous cystadenomas (13 cases), borderline serous tumor (23 cases), or serous carcinoma (64 cases) were identified in the archive scan, and sections were taken from formaline fixed paraffin embeded blocks containing a sufficient amount of tumor. The study was approved by Inonu University Ethics Committee Board.

Immunohistochemistry

Paraffin-embedded tissues with a diameter of 5mm from tumor areas were extracted for immunohistochemical examination. Twenty tissues were re-blocked in a single block. Immunohistochemistry was performed on 4-µm freshly cut sections from paraffin-embedded tissue blocks with using fully automated system ("Dako Omnis", Agilent, US) and anti FGFR1 (mouse monoclonal, M2F12, 1:25 dilution, heat-pretreatment for epitope retrieval in EDTA buffer, incubation at 37°C for 40 minutes, Medaysis, US). Immunohistochemically stained preparations were evaluated under a light microscope by a pathologist and a pathology resident. The cytoplasmic or membranous staining pattern in tumor cells was considered significant, and the extent and intensity of the staining were evaluated separately.

Staining intensity in tumor cells was evaluated as follows; no staining = 0 points, weak staining = 1 point, medium staining = 2 points, and strong staining = 3 points. The prevalence of stained tumor cells was calculated as a percentage (%) obtained by proportioning the total number of cells. H scores were obtained by multiplying the percentage (%) values determined for prevalence and the scores given for intensity. In this system, <1% positive cells is considered to be a negative result. According to Dabbs et al., H-score has a broader dynamic range [6].

Staining of normal breast tissue was taken as reference as the external positive control.

PCR

Reverse transcription reactions were carried out with RiboEx kit (GeneAll Biotechnology, Korea, Catalog no: 301-001). cDNA synthesis was performed using the HyberScriptTM First strand synthesis kit (GeneAll Biotechnology, Korea, Catalog no: 601-005). Quantitative RT-PCR was performed in triplicate by real-time PCR using RealAmpTM SYBR qPCR Master kit (GeneAll Biotechnology, Korea, Catalog no: 801-051) on a 7500 Fast detection system (Applied Biosystems). Primer sequences were as follows:

FGFR1 5'-AATGAGTACGGCAGCATCAAC-3'and
5'-ACCTCGATGTGCTTTAGCCAC-3';

ACTB 5'-CATGTACGTTGCTATCCAGGC-3'and
5'-CTCCTTAATGTCACGCACGAT-3'.

The thermal cycler conditions were as follows: 95°C for 300 sec, followed by a two-step PCR of 40 cycles at 95°C for 15 sec and 55-68°C for 60 sec. Quality of the RNA was analyzed on the Qubit 4 Fluorometer using the Quant-iT™ RNA Assay Kits and Quant-iT RNA HS Reagent (cat.no Q33140) kit. The RNA was identified with high quality and sensitive reading, and its separation from the protein was observed. Confirmed with a melt curve. Relative mRNA levels were determined using the$\Delta\Delta$Ct method. Values were expressed relative to ACTB.

Statistical analysis

The analyses were evaluated in IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY: IBM Corp.). When calculating the sample of the study, G*Power 3.1.9.2 program was used and the "Increased FGFR1 copy number in lung squamous cell carcinomas" study was taken as reference. Accordingly, it was determined that a total of at least 84 people should be reached, with a 95% confidence interval, 80% power and an allocation ratio of 6, at least 12 controls and 72 patients. In the study, descriptive data are shown as n, % values in categorical data and as median interquartile range (25-75 percentile values) values in continuous data. Chi-square analysis (Pearson Chi-square) was applied in the comparison of categorical variables between groups. The compliance of continuous variables with normal distribution was evaluated with the Kolmogorov-Smirnov test. Kruskal Wallis test with Bonferroni post-hoc correction was used in the comparison of more than two variables. Spearman correlation test was used in the examination of the relationship between continuous variables. Statistical significance level was accepted as $p<0.05$ in the analyses.

Results

Clinicopathological parameters

Of the 100 cases included in our study, 59 were diagnosed with HGSOC, 5 were LGSOC, 28 were BST and 13 were serous cystadenoma. The age range of the patients was 18-88, and the mean age was 44 for SCs, 38 for BSTs, 57 for LGSOCs, 56 for HGSOCs, and 51 for all tumors.

According to the 2020 FIGO staging system; 29 cases in stage 1, 8 cases in stage 2, 38 cases in stage 3, 8 cases in stage 4. Data to determine the stage of 4 cases could not be reached.

The majority of HGSOC cases are in the advanced stage (FIGO stage 3-4), BST and LGSOC cases are in the early stage. 8 (14.3%) of HGSOC cases were stage 4, 37 (66.1%) stage 3, 6 (10.7%) stage 2, 5 (8.9%) stage 1; 1 (3.7%) of LGSOC/BST cases were stage 3, 2 (7.4%) stage 2, 24 (88.9%) stage 1 (Table 1).

Table 1. FIGO stage, PCR fold change levels and Immunohistochemistry H scores by diagnostic groups.

		HGSOC		LGSOC/BST		SC		p
		n	%	n	%	n	%	
FIGO	Stage 1	5	8.9	24	88.9	-		**<0.001***
	Stage 2	6	10.7	2	7.4			
	Stage 3	37	66.1	1	3.7			
	Stage 4	8	14.3	0	.0			
PCR		6.3 (4.0-9.0)[a]		1.1 (.6-2.4)[b]		1.2 (.7-1.3)[b]		**<0.001***
Immunohistochemistry-cytoplasmic H score		200.0 (100.0-300.0)[a]		100.0 (100.0-200.0)[b]		80.0 (60.0-90.0)[c]		**<0.001***

* Chi-square analysis, **Kruskal Wallis test was applied. [a,b,c]Group from which the difference originates.

Table 2. PCR fold change levels and Immunohistochemistry H scores by FIGO stages.

	PCR Median (IQR)	p*	IHK Median (IQR)	p*
Stage 1	1.3 (.6-7.0)[a]	**<0.001**	100.0 (100.0-200.0)[a]	**0.015**
Stage 2	4.1 (2.6-7.2)[a,b]		150.0 (87.5-250.0)[a,b]	
Stage 3	6.8 (3.6-9.5)[b]		200.0 (200.0-300.0)[b]	
Stage 4	4.7 (4.1-6.5)[a,b]		200.0 (120.0-250.0)[a,b]	

*Kruskal Wallis test was applied. [a,b]Group from which the difference originated.

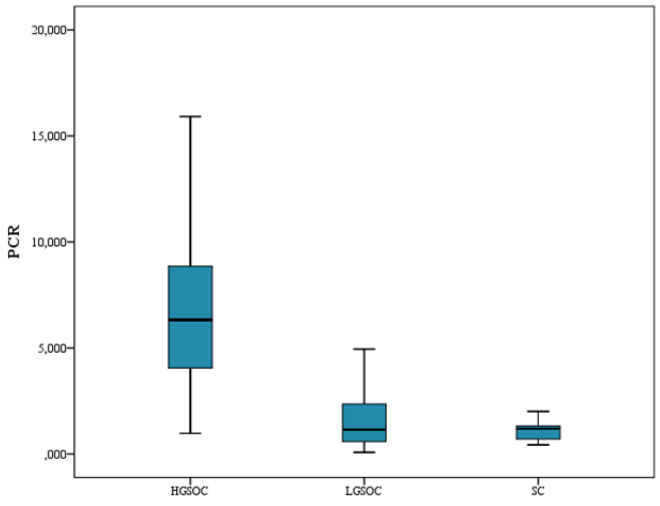

Figure 1. FGFR1 fold changes by groups.

When the FIGO stages of HGSOC, LGSOC and borderline tumors were compared, it was seen that HGSOC had a relatively advanced FIGO stage to borderline tumors, as expected. There was no significant difference in the FIGO stage between LGSOC and HGSOC and borderline tumors. It was thought that this situation might be due to the low number of LGCC cases.

Gene expression analysis

RT-PCR analyzes were performed by obtaining cDNA from the samples of the cases. FGFR1 and ACTB gene

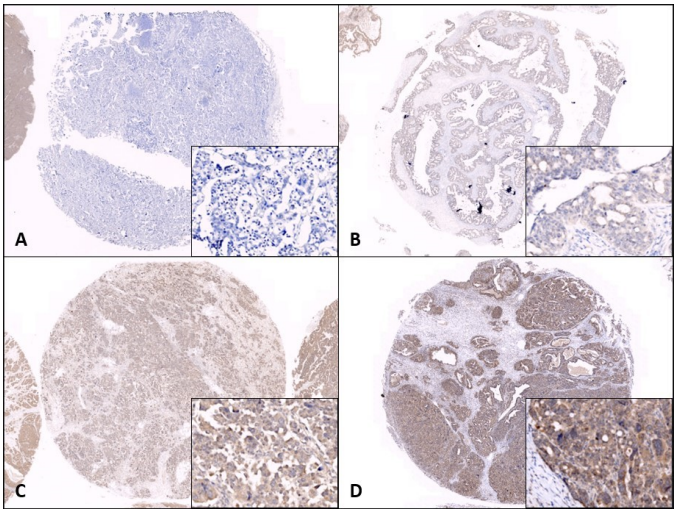

Figure 2. Different degrees of cytoplasmic staining with FGFR1 immunohistochemistry stain in HGSOCs. Diffuse cytoplasmic staining was observed in most of the tumors. Negative (A). Weak (+) staining (B). Moderate (++) staining (C). Strong (+++) staining (D).

regions for each patient were studied by repeating 3 times in the Applied BiosystemsTM 7500 Fast Real-Time PCR device. The cases were divided into 3 main groups as SC, LGSOC and Borderline, and HGSOC. SC group considered as control cases. We determined the cut-off ratio of 1 in FGFR1 expression, which is the mean folding change of serous cystadenomas. While no increase in FGFR1 expression was detected in 1 (1.7%) of 59 HGSOC cases, FGFR1 expression was increased in 58 (98.3%) of them. These numbers are 12 (42.9%) and 16 (57.1%) in LGSOC/Borderline group, respectively; in the SC group, it was found to be 6 (46.2%) and 7 (53.8%). There is a significant difference between the groups in terms of PCR and this difference is due to the difference between the HGSOC group and the other two groups, and the PCR value of the HGSOC group is higher ($p<0.001$) (Table 1).

FIGO stage data were available for 83 of the cases. When grouped according to FIGO stages, the median FGFR1 fold changes were found to be 1.3 for FIGO stage 1, 4.1 for FIGO stage 2, 6.8 for FIGO stage 3, and 4.7 for FIGO stage 4, respectively. A significant difference was observed

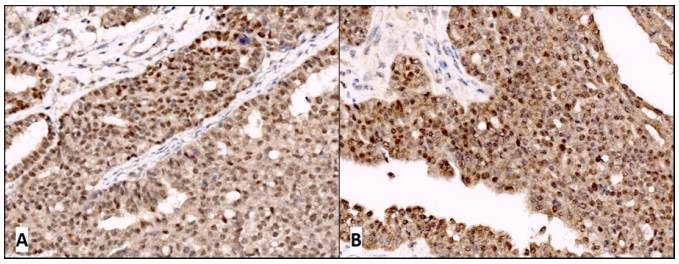

Figure 3. Nuclear staining pattern seen in 2 cases with HGSOC (FGFR1, 100x).

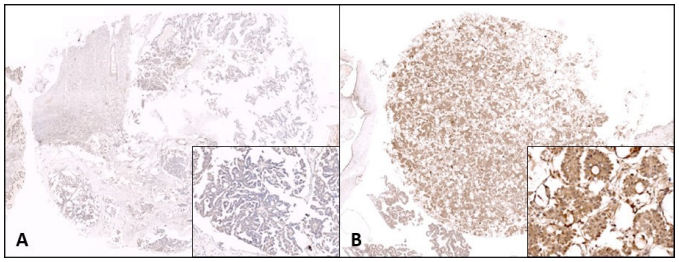

Figure 4. FGFR1 immunohistochemical staining in LGSOC cases. Diffuse weak (+) cytoplasmic staining (A). Diffuse strong (+++) cytoplasmic staining (B).

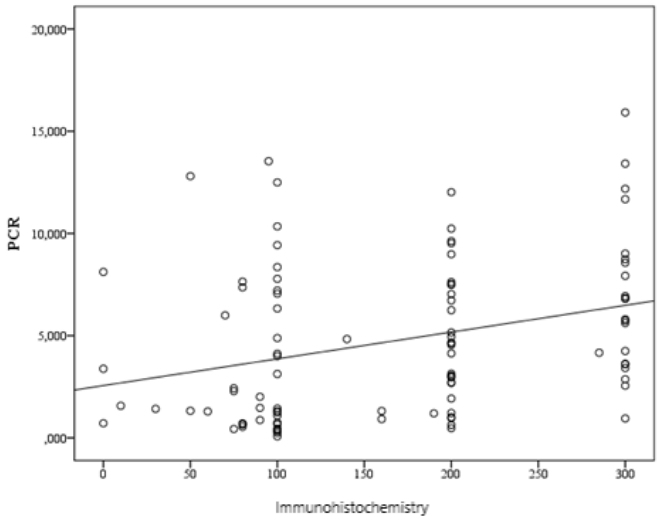

Figure 5. Correlation between immunohistochemistry and PCR.

between the FIGO stages in terms of PCR ($p<0.001$), and this difference originated only from the difference between Stage 1 and Stage 3, and Stage 1 was the lowest (Table 2).

Immunohistochemistry

Immunohistochemical staining intensity and percentage calculations were made in all cases. Diffuse cytoplasmic staining pattern was observed in most of the tumors with different intensities (Figure 2). Nuclear staining was observed in 85% and 20% prevalences in only 2 HGSOC cases (Figure 3).

Although the prevalence of staining in HGSOC, LGSOC and BST cases is generally in the range of 90-100%, it was evaluated as 75-80% in rare cases. Strong (+++) cytoplasmic staining was observed in 1 of 5 cases diagnosed with LGSOC, and weak (+) cytoplasmic staining was observed in the other 4 cases. Out of 23 cases with borderline serous tumor, 9 cases showed moderate (++) cytoplasmic staining and 14 cases showed weak (+) cytoplasmic staining (Figure 4).

A significant difference was observed between the FIGO stages in terms of PCR ($p<0.001$) and IHC score ($p=0.015$), and this difference originated only from the difference between Stage 1 and Stage 3, and Stage 1 was the lowest (Table 2).

The cytoplasmic H scores for HGSOC, LGSOC/Borderline and SC groups were found to be 200.0, 100.0 and 80.0, respectively, according to the diagnostic groups. When nuclear and cytoplasmic stainings were examined, cytoplasmic staining was thought to be significant and statistical evaluations were made according to cytoplasmic staining and the H score of this staining. When cytoplasmic H scores were examined statistically according to the diagnosis groups, all groups were found to be statistically significantly different from each other ($p<0.001$) (Table 1).

In the correlation analysis, a positive significant correlation was observed between PCR and IHC ($r=0.341$; $p=0.001$) (Figure 5).

Discussion

In addition to the histomorphological and immunohistochemical classifications in ovarian cancer, it was determined that tumors developed from different pathways by molecular findings, and a new classification was proposed according to their molecular characteristics. Epithelial tumors are grouped as Type 1 and Type 2 tumors, and the cell groups, precursor lesions and prognostic information of these groups are categorized and compared. BRAF and KRAS mutations are high in type 1 tumors and they do not show TP53 mutations. Type 2 tumors show TP53 mutation in almost all cases [2, 14].

When the studies on FGFR1 expression in ovarian tumors were searched in the literature, Valve et al. in their study on 51 ovarian tumors, it was observed that an expression increase of 91% was detected in 24 serous carcinoma cases with RT-PCR [7]. Birrer et al. In the study, FGF1 amplification was detected in 66% of 42 HGSOC cases with RT-PCR, and increased FGF1 mRNA expression was associated with a poor prognosis [15]. In the study conducted by Helsten et al. with the new generation sequencing method, the rate of FGFR1 amplification in 233 ovarian tumors was reported as 5-9%, and no subtype information was given [4]. In our study, the rate of FGFR1 amplification (95%) in serous ovarian carcinomas was found to be higher than other studies. When we look at the amplification rates, we found 98.31% amplification in the HGSOC group and 57.14% in the Borderline/LGSOC group. When we evaluated the fold change rates, it was seen that there was a statistically significant increase in favor of HGSOC in the HGSOC group compared to the Borderline/LGSOC and SC groups.

In the study by Cole et al., it was shown that the antitumor activity of cisplatin increased when FGFR1 inhibition was applied together with cisplatin in 10 serous

ovarian carcinomas and 5 normal ovaries by in situ hybridization method, and in 40 serous ovarian carcinomas and 10 normal ovaries by immunohistochemistry method. With cell cycle analyses, it was stated that this effect was achieved by increasing the apoptosis of both agents [14]. This provides support for the use of FGFR-1 as a target in therapy.

The effects of FGFR1 amplification have been demonstrated in many studies on other tumor types. FGFR1 expression in squamous cell lung carcinomas is directly proportional to lymph node metastasis [16], and FGFR1 expression is inversely proportional to chemosensitivity in small cell lung carcinomas [17]; it is an independent prognostic indicator in breast tumors [18], it increases tamoxifen sensitivity when used together with tamoxifen [19]; It has been proven that increased expression of FGFR receptors in diffuse gastric carcinomas is associated with depth of invasion, distant metastasis, histological grade, and recurrence [20].

It is known that BSTs with micropapillary morphology have a relatively poor prognosis compared to BSTs without micropapillary morphology. Micropapillary morphology was detected in 2 of the borderline serous tumor cases. When the FGFR1 fold changes of these cases were examined, it was seen that they were 7.06 and 4.55. The fact that these 2 cases have FGFR1 expression at the level of HGSOCs, different from the mean of BST cases and LGSOC cases, supports that micropapillary variant BSTs are a higher-grade tumor with a worse prognosis than other BST cases. Conditions such as ischemia, hemorrhage and necrosis can affect the amount of evaluable cells in the tissue and cause false negativity. In our study, hemorrhage and ischemia findings were observed in 1 LGSOC and 1 BST case. In the evaluation made with light microscope, it was seen that most of the cells in the tissue were not degenerated and unaffected. However, fold change values were found to be lower than expected for the relevant diagnosis group. This difference was thought to occur because the cells were affected by ischemia. Since 1 of the HGSOC cases had a fold change of less than 1, the clinical information and histological findings were re-examined. However, no additional clinical or pathological features were observed in the case. This case, whose FGFR1 amplification was not detected, was found to be ex within 1 month.

FGFR1 expression fold changes detected by RT-PCR in all cases were statistically correlated with immunohistochemically determined cytoplasmic H score (p=0.001). However, statistical significance could not be determined with positivity/negative criteria such as staining of more than 1% tumor cells, staining of more than 10% tumor cells or being considered positive when the H score is greater than 100 in previous publications [8–13].

Various studies are being conducted to create microarrays in epithelial ovarian tumors, and in our study, 1 core of 5 mm diameter was examined from 100 cases. Hecht et al. CK7, CK20, p53, WT-1, ER, PR, Ki-67 immunohistochemical stains were studied and compared with the whole block section in the study performed by taking 3 samples with a diameter of 0.6 mm from each of 174 epithelial ovarian tumors. When comparing single-core, two-cores and three-cores, respectively, it is reported that results with 91%, 95% and 96% accuracy are obtained. The tissue size (5 mm in diameter) we used in the microarray was found to be sufficient to perform immunohistochemical studies with the support of the data in the literature. It is emphasized that antigenicity may be affected as the age of paraffin block progresses (grouping as >10 years and ≤10 years) and the importance of site selection in borderline tumors [21]. In our study, the oldest paraffin block in terms of tissue age was 10 years ago. On microarray, in the study of Permuth et al., complete block section examination in 59 epithelial ovarian cancers and 1 core examination of 1 mm diameter taken from the central region were compared in terms of mismatch repair (MMR) gene expression loss. In 17 cases with loss of MMR gene expression in the central chord, 5 more cores of 1 mm diameter are sampled from the peripheral region. In 11 of these cases, there is loss of expression in the center, while expression is detected in the periphery. It is interpreted that this situation may be related to fixation [22]. In our study, relevant areas from H&E sections were selected considering fixation and staining in previous immunohistochemical staining. Tissue was taken from subcapsular areas in appropriate cases. Tissue integrity was confirmed by H&E re-staining in sections taken after the immunohistochemistry method.

As a result of the immunohistochemical examination performed with the FGFR1 antibody, it was determined that the cytoplasmic staining decreased, respectively, and was observed at varying rates in the HGSOC, LGSOC/Borderline and SC groups. In this regard, there are not enough studies on ovarian carcinomas with the FGFR1 immunohistochemistry method in the literature.

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Ethical approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Malatya Clinical Researchs (Date 2019 / No 182).

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Mehmet Ozcan. The first draft of the manuscript was written by Mehmet Ozcan and other authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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