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Original Article

Combined use of WNT signal pathway inhibitor FH535 and docetaxel causes mitotic catastrophism and antiproliferative effect in non-small cell lung cancer

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Abstract

The development of treatment methods used in the treatment of non-small cell lung cancer (NSCLC) is important to prevent problem of increasing mortality. However, the treatment methods used in clinical settings at the clinic are insufficient to eliminate this problem. For this purpose, it was aimed to determine whether the combination of docetaxel (DTX) and FH535 can be used as an anticancer agent candidate in A549 cells and whether it is a candidate drug combination that can be used in clinical treatment after in vivo studies. FH535 is a WNT signaling pathway inhibitor and is known to be overactive in NSCLC. In this study, the effects of DTX and WNT signaling pathway inhibitor FH535 used in NSCLC treatment on A549 and BEAS-2B cell lines were evaluated at the cellular level. While increasing the anticancer activity in A549 cells, the doses showing minimum toxic effect in BEAS-2B cells were determined by Real Time Cell Analysis method. Mitotic activity, BrdU cell proliferation assay and caspase 3,7 activity assay were performed for detailed analysis of the combination dose at cellular level. The results show that the combined dose had an antimitotic effect on A549 cells, causing mitotic catastrophism, while in BEAS-2B cells neither agent was more toxic than either agent alone, reducing mitotic activity and BrdU activity, leading the cell to mitotic catastrophism, while caspase 3,7 activity was unchanged. This study demonstrated for the first time the effects of the combination of DTX and FH535 on A549 and BEAS-2B cell lines.

Keywords: A549, BEAS-2B, DTX, FH535, WNT signaling pathway, NSCLC.

1. Introduction

Cancer is the second leading cause of death in the world and remains a critical global health challenge, necessitating innovative approaches in targeted drug delivery systems to enhance therapeutic efficacy and patient outcomes [1]. In a study, according to the rate and demographic changes observed between 2006 and 2010, in the ranking of mortality rates of cancer types between 2020 and 2030, it is predicted that the cancers with the highest mortality rates will be lung, pancreatic, and liver cancer [2]. According to a study that compared 36 different types of cancer in 185 countries, lung cancer, which is the most common cancer among the 9.6 million cancer cases worldwide, has been determined as the type of cancer that causes the most deaths, with a mortality rate of 18.4% [3].

As a result of statistical studies, the average 5-year survival rate of lung cancer patients worldwide was determined to be 21.7% [4]. Since most of the factors that cause lung cancer cannot be fully elucidated, deficiencies in the diagnosis, prognosis, and treatment of the disease cannot prevent increased mortality in clinical practice [5]. It is important to understand the cellular pathways that play a role in the molecular mechanism of the disease and to develop new methods of treatment by targeting the activation and inhibition mechanisms of these pathways [6]. In this context, treatment methods for lung cancer have been developed through studies on drugs, drug combinations, or targeted molecules. Various studies have been conducted on targeted therapy, especially for the treatment of non-small-cell lung cancer [7]. In 2015, the World Health Organization (WHO) divided lung cancer into two groups: small-cell lung cancer (SCLC) and non-small-cell lung cancer [8]. NSCLC accounts for 80–85% of all lung cancers [9]. Since the symptoms are detected late in NSCLC, patients do not have the chance to undergo surgery because of the delayed diagnosis [10].

DTX, a molecule belonging to the taxane class, is a semisynthetic antineoplastic agent obtained by isolating it from the needle-tipped leaves of *Taxus baccata* L. and used in various cancer treatments [11]. It is thought to block the G_2/M phase of the cell cycle by depolymerizing the microtubules, thus inhibiting cell proliferation and leading to cell death [12].

The WNT signaling pathway is known to have multiple functions, including cell proliferation, survival, self-renewal, cell growth, movement, and differentiation [13].

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Targeting the WNT signaling pathway in cancer treatment, inhibiting tumor growth with minimal effect on somatic cells, inhibiting tumor recurrence, and preventing the development of resistance to chemotherapy and radiotherapy. For this reason, WNT signaling pathway inhibitors are attracting the attention of many researchers, and the development of different inhibitors continues. One such inhibitor, FH535, is a specific β-catenin inhibitor. β-catenin uptake and antagonizes transcription mediated by β-catenin/TCF [14].

In this study, the effects of the combined use of DTX and WNT signaling pathway inhibitor FH535 used in the treatment of NSCLC on adenocarcinomic human alveolar basal epithelium cell A549 and healthy bronchial epithelial cell BEAS-2B cell lines were evaluated by MTT and real-time cell analysis. A combined dose was determined in A549 cells that showed minimal damage to BEAS-2B cells while reducing cancer cell activity. The effects of this dose at the cellular level were determined *in vitro* using mitotic activity, BrdU cell proliferation, and caspase 3,7 activity analysis. In this context, it is aimed to create a new treatment protocol premise that is thought to give important results for use in clinical treatment with the results obtained from more detailed in vitro and then *in vivo* experiments.

2. Materials and Methods

2.1. Cell lines and culture

A549 and BEAS-2B cells used in the experiments were purchased by our research laboratory from the American Type Culture Collection (ATTC, VA, USA). The cells were supplemented with 10% (v/v) fetal bovine serum (FBS; pan Biotech, cat. no. p04-3306), 100 IU/ml penicillin (Pronapen, Pfizer), and RPMI-1640 (Sigma, cat. no. r6504) containing 100 µg/ml streptomycin (streptomycin sulfate, I.E. Ulugay) with 4.4% $NaHCO_3$ and a pH of 7.2. The cells were maintained at 37°C in an atmosphere of 95% humidity and 5% CO_2.

2.2. Real-Time Cell analysis

In xCELLigence DP (RTCA, xCELLigence, Roche), an E-plate with 16 wells was used. In the experimental process for the background measurement, 100 µl of the medium was added to each well. Cells were seeded in each well at 8×10^3 cells/ml and A549 cells and 5×10^3 cells/ml in BEAS-2B cells. The final volume was 200 µl. After seeding, E-plates were incubated at room temperature in a sterile working cabinet for 20 min. The incubation was continued at 37°C and 5% CO_2. The device was set up to perform cell analysis every 15 min for 100 h. Subsequently, determined doses were added, and time-dependent graphs were obtained by continuing to take measurements. These steps were repeated separately for both cell lines for the DTX, FH535, and DTX+FH535 combinations.

2.3. Mitotic activity analysis

The Mitotic Assay Kit was used for 24, 48, and 72-hour experiments in the A549 cell line. In each well of a 96-well plate, 15×10^4 cells/ml were seeded after 24 h of incubation in a medium containing 95% air and 5% CO_2 at 37°C. The Mitotic Assay Kit (Colorimetric and Chemiluminescent, Active Motif, cat. no.18021) protocol was completed in accordance with the protocol.

2.4. BrdU activity analysis

The BrdU Assay Kit was used for 24, 48, and 72-hour experiments in the A549 cell line. In each well of a 96-well plate, 15×10^4 cells/ml were seeded after 24 h of incubation in a medium containing 95% air and 5% CO_2 at 37°C. The BrdU Assay Kit (Millipore, cat. no. 2750) protocol was completed in accordance with the protocol.

2.5. Caspase 3,7 activity analysis

The CaspaTag Caspase-3,7 In Situ Assay Kit Fluorescein™ Assay Kit was used for 24, 48, and 72-hour experiments in the A549 cell line. In each well of a 96-well plate, 15×10^4 cells/ml were seeded after 24 h of incubation in a medium containing 95% air and 5% CO_2 at 37°C. CaspaTag Caspase-3,7 In Situ Assay Kit Fluorescein™ (Millipore, cat. no. apt423) protocol was completed in accordance with the protocol.

2.6. Statistical analysis

All experiments were repeated three times. Data from the experimental groups were compared using a one-way ANOVA test. Instead of comparing all groups in pairs and multifaceted in the experiment, the DUNNET'S test used the control group to measure the importance of experimental group, and the t-test assessed the significance of the experimental group. Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, California, USA) has been done. A $P<0.05$ significance level was accepted in the tests.

3. Results

3.1 Analysis of DTX, FH535, and DTX+FH535 effect with real-time cell analysis system in A549 and BEAS-2B cells

The effects of the agents applied to both cell lines were determined by measuring at intervals of 15 min for 100 hours. According to xCELLigence real-time cell analysis data, DTX applied to A549 cells alone was effective compared to the control group, but when BEAS-2B was administered alone, it damaged the cells (Fig. 1A, 2A). FH535 caused 50% cell death in A549 cells and almost no toxic effects in BEAS-2B cells. The determined appropriate and effective dosages were minimized to reduce the toxic effect of the agents applied to A549 and BEAS-2B cell lines in combined doses, and their effects at the cellular level were examined by measuring at 15-minute intervals for 100 hours (Fig. 1B, 2B). In A549 cells, the IC_{50} values for DTX were 1 µM and for FH535 this value was 20 µM. To determine the doses that had the least harmful effect on BEAS-2B cells and to provide the desired effect in A549 cells, 1 µM DTX and 5 µM DTX were applied to BEAS-2B cells. As both doses showed the same toxicity, the combined study with the lowest dose of 1 µM DTX and lower doses of DTX was continued. As a result of the evaluations, it was determined that the dose with the most effect on A549 was the dose consisting of a combination of 0.005 µM DTX+0.5 µM FH535 and that this dose showed an anti-mitotic effect on A549 while showing a minimal harmful effect on BEAS-2B (Fig. 1C, 2C).

With the doses of FH535 given to the BEAS-2B cells, 30 µM FH535 and 40 µM FH535 were used to determine the effect at the maximum dosage given, and it was found that FH535 caused little damage to healthy lung cells at high doses. Therefore, it was observed that a high dose

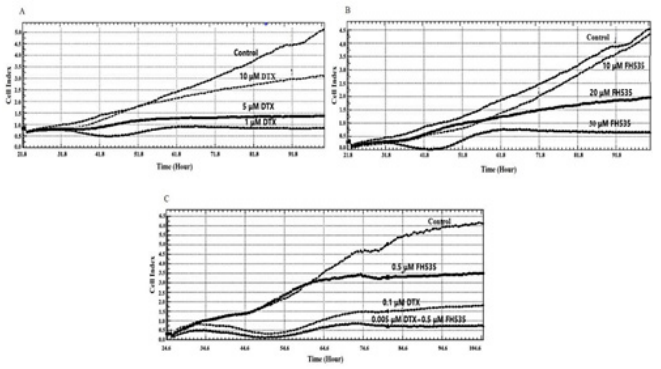

Fig. 1. Graph of real-time cell analysis of A549 cells treated with DTX, FH535 and DTX+FH535. A: Graph of real-time cell analysis of A549 cells treated with DTX at concentrations of 1 µM, 5 µM, and 10 µM for 100 hours. B: Graph of real-time cell analysis of A549 cells treated with FH535 at concentrations of 10 µM, 20 µM, and 30 µM for 100 hours.. C: Graph of real-time cell analysis of A549 cells treated with DTX, FH535, and DTX+FH535 for concentrations of 0.5 µM FH535, 0.1 µM DTX and 0.005 µM DTX+0.5 µM FH535 for 100-hours.

of FH535 did not harm healthy cells and showed a lethal effect as the dose increased in cancer cells. However, since the patient's exposure to minimal chemical agents in the treatment protocols is important for the health of the organism, the experiments were continued over the FH535 dose determined as 0.5 µM in the study.

The results indicated that the combined dose showed greater efficacy in A549 cells than in the control group. The same combined dose in BEAS-2B caused damage equivalent to that induced by DTX alone. Based on these results, the DTX+FH535 combination, which is a combination agent candidate that does not cause more harm than DTX to BEAS-2B, which is frequently used in cancer treatment and shows greater efficacy in A549 than DTX alone, was found to be successful. The results of the time-dependent cell index graph indicated that the combination of cancer cells was effective in demonstrating antimitotic activity. The combination index calculation for 50% toxicity, which is used to achieve more effective results in combination therapies, was performed according to the following formula:

$CI = (D)_1/(DX)_1 + (D)_2/(DX)_2 + \alpha(D)_1.(D)_2/(DX)_1.(DX)_2$

$(D)_1$: A dose of drug 1 to produce 50% cell kill in with $(D)_2$

$(D)_2$: A dose of drug 2 to produce 50% cell kill in with $(D)_1$

$(DX)_1$: A dose of drug 1 to produce 50% cell kill alone

$(DX)_2$: A dose of drug 2 to produce 50% cell kill alone

α: "0" if one of the two drugs is present and the other does not work; is considered "1" if two drugs act together, and the calculated value; CI > 1.3 indicates antagonism, CI = 1.1–1.3 indicates medium antagonism, CI =0.9-1.1 indicates supportive effect, CI = 0.8-0.9 indicates mild synergy, CI= 0.6-0.8 indicates medium synergy, CI = 0.4–0.6 shows synergy and CI= 0.2-0.4 indicates high synergism [15].

The combined dose used in this study on A549 cells has a high synergistic effect, with a combination index value of 0.37505. This result indicates that the determined combination dose has a higher effect than the DTX and FH535 doses administered separately, so the combined dose applied to the A549 cell line is more effective than the use of either agent alone.

3.2. The DTX and FH535 combination in the A549 cell contributes to mitotic catastrophe by reducing mitotic activity

The combined dose decreased mitotic activity in A549 cells at 24 and 72 h and led to cell death through a mitotic catastrophe (Figure 3A). Mitotic activity, which decreases and changes over time, has supported that combined agent application leads to the cell death type called mitotic catastrophe, and in line with real-time cell analysis, the combined application has an antimitotic effect on cancer cells. In mitotic catastrophism, the cell dies in metaphase or before it enters metaphase as a result of DNA damage at the checkpoints of cell division during mitosis. Mitosis stops and degradation occurs in the mitotic spindle strands [16]. The results obtained show that FH535 also has a supportive effect on this effect of DTX, which prevents microtubule depolymerization by binding to the β-tubulin subunit of tubulin heterodimers and inhibits microtubule

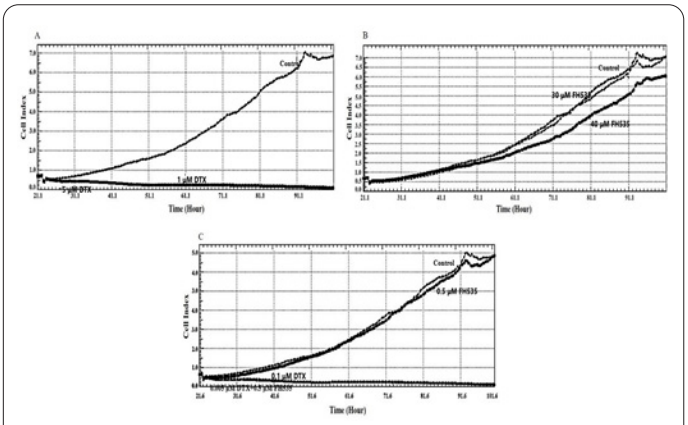

Fig. 2. Graph of real-time cell analysis of BEAS-2B cells treated with DTX, FH535 and DTX+FH535. A: Graph of real-time cell analysis of BEAS-2B cells treated with DTX at concentrations of 1 µM and 5 µM for 100 hours. B: Graph of real-time cell analysis of BEAS-2B cells treated with FH535 at concentrations of 30 µM and 40 µM for 100 hours. C: Graph of real-time cell analysis of BEAS-2B cells treated with DTX, FH535, and DTX+FH535 at concentrations of 0.5 µM FH535, 0.1 µM DTX and 0.005 µM DTX+0.5 µM FH535 for 100-hours.

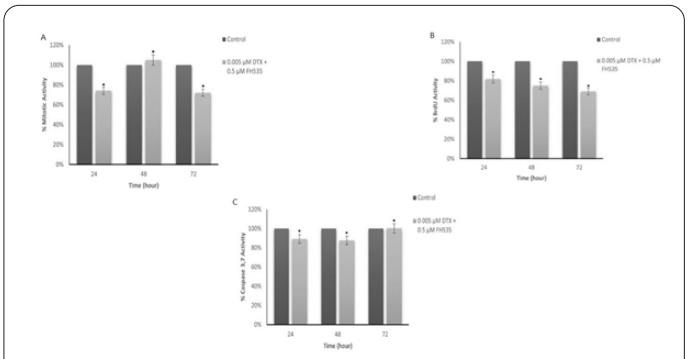

Fig. 3. Graph of mitotic activity, BrdU activity and caspase 3,7 activity of A549 cell treated with A549+FH535. A: Graph of mitotic activity of A549 cell treated with a combined dose of 0.005 µM DTX+0.5 µM FH535 (*p<0.05). B: Graph of BrdU activity of A549 cell treated with a combined dose of 0.005 µM DTX+0.5 µM FH535 (*p<0.05). C: Graph of caspase 3,7 activity of A549 cell treated with a combined dose of 0.005 µM DTX+0.5 µM FH535(*p<0.05).

depolymerization, and in this way, this combination reduces mitosis division and leads the cell to death by dragging it to mitotic catastrophism.

3.3. DTX and FH535 combination in A549 reduces BrdU cell proliferation activity

The decrease in BrdU activity at 24 and 48 h, and most significantly at 72-hours compared to the control group, supports our idea that when evaluated by the findings of mitotic activity, there is a decrease in the synthesis phase of the cell cycle and the number of cells, and therefore goes to mitotic catastrophism. This indicates that the BrdU activity of the cancer cells treated with the combined drug at 24, 48, and 72-hours decreased to 68% in 72-hours compared to the control group depending on the time, and that the number of cells proliferated decreased with the decrease in mitosis division in parallel with the results of the mitotic activity analysis (Fig. 3B).

3.4. DTX+FH535 combination in A549 does not affect caspase 3-7 activity

There was no significant change in caspase 3,7 activity at 24, 48, and 72 hours following combined dose administration compared to the control group. This result supports the idea that, based on the data obtained from real-time cell analysis, the cancer cells to which the combined dose was administered did not die by apoptotic cell death, but by an antimitotic effect and therefore by mitotic catastrophe (Fig. 3C).

4. Discussion

It has been supported by many studies that disorders in the WNT signaling pathway are related to the pathogenesis of various diseases, especially cancer [17–19]. In studies, active WNT signaling or over-expression of *WNT-1* has been seen in most of the NSCLC cell lines [20–23]. It is thought that this signaling pathway plays an active role in cellular processes, such as cell proliferation and movement; therefore, any factor that disrupts the homeostasis of these processes may also affect carcinogenesis. Disorder WNT signaling is effective in the formation of various cancers, such as colon, lung, breast, thyroid, prostate, and blood cancer [24–28]. A study of A549, H1703, H460, and H838 NSCLC cell lines showed that WNT-1 protein is overexpressed in these cell lines and that when the expression of these proteins is inhibited, the cells enter the apoptotic death pathway [20]. Another study indicated that the Dvl protein, which is involved in this pathway, is also associated with Rac and Rho proteins, which are known to be overexpressed in NSCLC and play an active role in lung cancer, and may therefore be effective in the pathogenesis of lung cancer [29, 30]. Overactivation of the WNT signaling pathway in NSCLC has led researchers to discover pathways or molecules that would inhibit this activation. Of these inhibitors, FH535 inhibits the proliferation of hepatocellular carcinoma stem cells and glioma and stomach cancer cells, increases programmed cell death in myeloid leukemia, reduces the number of cells in pancreatic cancer, inhibits cell growth in osteosarcoma, suppresses angiogenesis in colon cancer, and induces apoptosis by decreasing the expression of *cyclin D1* in hepatocellular carcinoma cells [31–38]. The mechanism by which FH535 inhibits the WNT signaling pathway is not yet known. In a study using FH535 in osteosarcoma, it was revealed that FH535 regulates the WNT signaling pathway by inhibiting tankyrase 1/2 (TNKS1/2) enzymes that prevent telomeric rebinding factor 1 from binding to DNA, which is one of the mechanisms used by the inhibitor to exert its effect [33]. *In vitro* studies have indicated that it has a shrinkage effect on gastric cancer cells, reduces cell density, and causes cell death [39]. In a study on leukemia, FH535 was shown to be the most effective WNT signaling pathway suppressor by comparative administration of the WNT signaling pathway inhibitors AV939, IWP2, and FH535 to cells [34]. A study on liver cancer stem cells and hepatocellular carcinoma cell lines showed that this inhibitor reduced the expression of *cyclin D1* and *survivin* [40]. In a study using HepG2 cells, as a result of treatment with FH535, it was seen that the proliferation of the cells was inhibited, and this inhibition was associated with a decrease in the expression of the β-catenin protein [18]. FH535 has been shown to significantly inhibit the growth, migration, and invasion of MDA-MB231 and HCC38 cells [41]. It has been shown to inhibit the proliferation and migration of DLD-1 and SW620 cell lines in colorectal cancer by significantly inducing G_2/M arrest [42]. In our study, we demonstrated that the WNT inhibitor FH535 showed anti-cancer properties in A549 cells, in agreement with previous studies.

In addition, it has been shown that there is almost no harm to healthy lung cells when used alone and that anticancer properties increase when used in combination with DTX. In addition, it did not cause more damage to the healthy lung cells, BEAS-2B, than DTX alone. The antiproliferative effect of this combination and FH535 on A549 in its single form has not been previously discovered in studies in the literature (Fig. 4).

The combination of docetaxel (DTX) and the WNT signaling pathway inhibitor FH535 presents a promising approach to enhance treatment efficacy in non-small cell lung cancer (NSCLC). The overactivity of the WNT pathway has been implicated in NSCLC tumorigenesis, making its inhibition a potential therapeutic strategy. Research indicates that WNT signaling plays a significant role in cancer cell proliferation and resistance to therapies, including DTX (11,13).

In the study at hand, the combined use of DTX and FH535 was shown to induce mitotic catastrophism in A549 cells, a hallmark of effective anticancer activity. This effect is consistent with findings from Roy et al. (2025), which

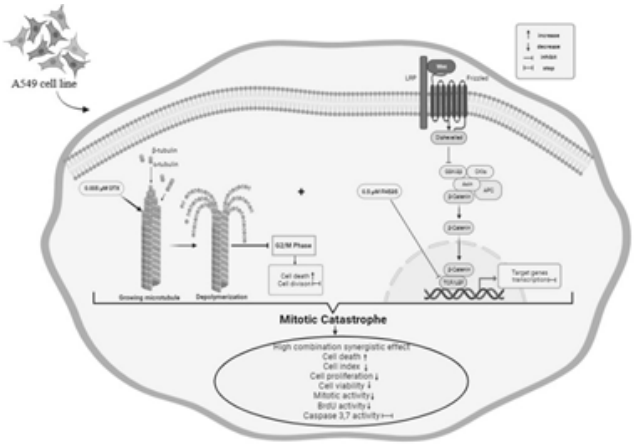

Fig. 4. Schematic version of the effect of the combined dose applied to cell A549.

discuss the role of ubiquitin ligases in cancer progression, highlighting that targeting specific pathways can enhance treatment outcomes [43]. Furthermore, the investigation into substrate stiffness by Ramezani et al. (2024) suggests that mechanical properties of the tumor microenvironment can influence drug efficacy, emphasizing the importance of understanding cellular context when evaluating treatment combinations [44].

Moreover, Yavuz et al. (2024) explored biomarkers in laryngeal cancer, indicating that circulating nucleic acids can provide insights into tumor behavior and response to therapy. This underscores the potential for similar biomarkers to be identified in NSCLC, which could aid in monitoring the effectiveness of DTX and FH535 combination therapy [45].

In this study, in which DTX and FH535 were administered in combination in A549 and BEAS-2B cells for the first time in the literature, FH535, which inhibits the overactivation of the WNT signaling pathway, which is thought to play a role in the pathogenesis of NSCLC, and DTX, which is frequently used in NSCLC treatment protocols, were administered in combination. This study, it was aimed to establish a new treatment protocol for *in vivo* and perhaps phase studies by determining the dose that will show antiproliferative effect on cancer cells while showing minimal damage to healthy cells. For this purpose, *in vitro* experiments showed that the combination of DTX+FH535 induced mitotic catastrophism in A549 cells, an NSCLC cell line, by showing an antimitotic effect and minimal damage to the healthy lung cell BEAS-2B, showing no more harmful effect than the cytotoxic effect of DTX alone. This combination dose was found to have a high synergistic effect with a combination index value of 0.37505, indicating a higher effect than that given by DTX and FH535 separately.

Declarations

Conflict of interest

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethical approval and consent to participate

Since cell lines were used in this study, ethical approval was not required.

Availability of data and material

All data generated or analyzed during this study are included in the article.

Code availability

Not applicable.

Author contributions

Eda Nur Avsar conceptualization (equal), data curation (equal), formal analysis (equal), investigation (lead), methodology (equal), software (lead), visualization (lead), validation (lead), writing-original draft preparation (lead), writing-review&editing (lead) of the manuscript. Idil Cetin conceptualization (equal), data curation (equal), formal analysis (equal), funding acquisition (lead), methodology (equal), project administration (lead), resources (lead), and supervision (lead) of the manuscript. Eda Nur Avsar and Idil Cetin confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

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