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Experimental Research

Combination effects of capecitabine, irinotecan and 17-AAG on colorectal cancer cell line (HT-29)

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ABSTRACT

Objevtive: Evasion of apoptosis is a major feature of cancer cells, therefore designing treatment strategies to target apoptotic pathways seems effective. In this study, we investigate the effect of 17-AAG (17-allylaminogeldanamycin) alone and in double and triple combination with capecitabine (Cap) and irinotecan (IR) on HT-29 colon cancer cell line apoptosis.

Methods: Capase-3, 8, 9, p53 and NF-κB genes expression were analyzed by Real-time PCR. DNA laddering assay was performed to confirm Real-time PCR results.

Results: Our results showed that all single treatment groups elevated expression of caspase-3, 8, and 9 significantly and IR/Cap was the only double combination group that could upregulate caspase-8 and -9. NF-κB was down-regulated in single treatment and IR/Cap double combination group, significantly. 17-AAG mono-treatment and IR/Cap and Cap/17-AAG double combination group significantly upregulated p53 gene expression.

Conclusion: Our findings showed proapoptotic effects of 17-AAG alone and in combination with Cap and IR. These findings propose 17-AAG in combination with routine chemotherapy, as a new protocol for colorectal cancer combination therapy.

1. Introduction

Colorectal cancer is the third most reported cancer and fourth cancer leading to cancer-causing mortaliy [1]. Cancer can be explained as a result of cancerous cells ability of apoptosis resistance and escape immune attack [2]. Apoptosis, the programmed cell death, is identified by DNA fragmentation, pyknosis and chromatin condensation [3]. Reduced expression and activation of caspase has an important role in cancer progression. In addition, p53 and MDM-2 (Mouse double minute 2 homolog), a p53 negative regulator, are effective components for targeted therapies, especially in stress-inducing therapeutics. Therefore, it is important to use chemotherapeutic agents that inhibit MDM-2 and repair wild-type or mutant p53 activity [4]. Studies have shown another vital factor in apoptosis regulation, NF-κB (nuclear factor kappa B), is upregulated in chemo-resistant tumors and NF-κB inhibition is associated with proapoptotic effects [5].

17-AAG (17-allylaminogeldanamycin) is a heat shock protein-90 (HSP-90) inhibitor. HSP-90 is a 90 kDa protein with important role in protecting the cell against stress stimuli, cell cycle regulation and apoptosis [6]. According to the anti-apoptotic effect of Bcr-Abl pathway and its chaperone association with HSP-90, it seems that 17-AAG can show proapoptotic effect [7]. There are some evidence suggested that intracellular Bcr-Abl levels down-regulated after 17-AAG treatment in human leukemia cells. In addition, current studies showed that 17-AAG treatment can activate caspase-9 and caspase-3 and induce apoptosis in K562 leukemia cells [8]. It was shown that in HCT-116 cells, 17-AAG and irinotecan alone could elevate caspase-3 gene and protein expression. A recent study has shown that 17-AAG along with irradiation and gold nanoparicles inhaces the activation of caspases 3, reduces cell vaiablity and promotes apoptosis in HCT-116 cells [9].

Abbreviations: 17-AAG, 17-allylaminogeldanamycin; Cap, Capesitapin.

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Common chemotherapeutic regimens include FOLFOX(5-FU/leucovorin and oxaliplatin) and FOLFIRI(5-FU/leucovorin and irinotecan) [10]. Capecitabine (Cap) is an oral agent that converts to 5FU by thymidine phosphorylase [11]. Irinotecan (CPT-11) is another chemotherapy agent that is converted by carboxylesterase to its active metabolite, SN-38, an inhibitor of topoisomerase I [12]. Despite great improvements in cancer chemotherapy, in some cases, clinically significant treatment outcomes are unachievable.

Given the role of caspase-3, 8, 9, p53 and NF-κB in apoptosis induction and lack of evidences regarding 17-AAG as the new therapeutic agent in colon cancer therapy, in this study we investigated 17-AAG, IR and Cap effects alone and in double and triple combination in HT-29, human colorectal cancer cell line.

2. Method and materials

2.1. Cell culture

Human colorectal adenocarcinoma cell line, HT-29, was obtained from Iranian Biological Resource Center (Tehran, Iran). HT-29 cells were cultured in high glucose DMEM (Dulbecco's Modified Eagle Medium) (Biowest), 10% fetal bovine serum and 1% penicillin-streptomycin 100X. Cells were grown at 37 °C and humid incubator with 5% CO2. HT-29s between 3 and 5 passages were used.

2.2. Cell viability assay

To assesses the cytotoxic effect of drugs on HT-29, Premix WST-1 Cell Proliferation Assay System (TAKARA BIO Inc., Japan) was used based on our previous study [13]. In brief, Cells were seeded in 96-well micro-plate. After 24 h incubation, the cells were exposed to various drugs concentrations; 17-AAG (80, 40, 20 and10 nM), IR (8, 4, 2, 1 and 0.5 μM) and Cap (8, 4, 2 and 1 μM). 24 h after exposure against the test compounds at 37 °C, cells were further incubated with WST-1 (10 μL WST-1 reagent) for 3 h at 37 °C. Absorbances were read at 450 nm with a reference wavelength >650 nm on Awareness Technology Stat Fax 2100 micro-plate reader (SAN DIEGO, CA, USA). Optical density (OD) values were obtained for control and treated cells. The inhibitory rate (%) was calculated using the following formula:

%viable cells = OD value of treated well / OD value of control well

The IC50 value of treatment groups relative to the control were calculated by Compusyn software (Combosyn, Inc., Paramus, USA). The IC50 values of Cap, IR and 17-AAG were calculated by WST-1 assay as 3.4 μM, 6.9 μM and 60 nM, respectively. All the experiments were repeated thrice and average value was obtained for analysis.

2.3. RNA extraction and cDNA synthesis

5×10^5 HT-29 cells were cultured in 6-well plates. Given that in our previous study, there were no significant differences between 24 h and 48 h treatment [13], we selected 24 h treatment in this study. After 24 h, cells were treated with IC50, 0.5 × IC50 and 0.25 × IC50, calculated in our previous study [13], in single drug-treated group, and double and triple treatment groups, respectively. Control samples that were not treated with the drug were included. 24 h after treatment cells were harvested and total RNA extraction was performed with GeneAll Hybrid-RTMRNA extraction kit (Korea). The integrity of extracted RNA was assessed with agarose gel electrophoresis and its purity was examined by measuring the ratio of optical density at 260nm/280 nm. GeneAllheperscript™ first strand synthesis kit (Korea) was used to synthesis of cDNA. 2 μL total RNA of each sample was reverse-transcribed into cDNA with random hexamer primers using Revert First-strand cDNA synthesis kit.

2.4. Real-time RT-PCR

Primer pairs shown in Table 1 were designed according to melting temperature and primer-dimer formation and were designed in NCBI/primer-blast (http://blast.ncbi.nlm.nih.gov/blast.cgi). After designing, primers were checked for primer dimer and hairpin formation with oligo7 software (http://www.generunner.net). Real-time PCR was performed using RealQ Plus 2x Master Mix Green (Amplicon, Denmark) and Bio Molecular Systems Mic qPCR cycler (QLD, Australia). The reaction was carried out in duplicate and PCR condition for all samples was as follow: 15 min at 95 °C, 40 cycles of the 20 s at 95 °C; 60 s at 58 °C using 2 μl of the cDNA. Expression levels of genes analyzed were normalized in accordance with the expression levels of β-actin as the housekeeping gene. Results were calculated by 2-ΔΔCt method.

2.5. DNA laddering assay

DNA of the treated and non-treated HT-29 cells was purified based on apoptotic DNA Laddering kit manufacturer's instructions (Roche Diagnostics, GmbH). Briefly, PBS and l lyses buffer (6 M guanidine-HCl, 10 mM Urea, 10 mM Tris-HCl, 20% Triton X-100 [v/v], pH 4.4) was added to cells and incubated for 10 min at 15–25 °C, after which isopropanol was added to the samples. The filter and collection tubes (provided in the kit) were combined and samples were pipetted into the upper reservoir. After centrifugation for 1 min at 2860×*g*, the eluate was discarded and the used collection tubes were combined with filters. A 500 μL washing buffer (20 mM NaCl; 2 mM Tris-HCl; 80% [v/v] ethanol: pH 7.5) was added and centrifuged at 2860×*g* for 1 min, and after discarding the collection tube, this washing phase was repeated and the residual wash buffer was removed by centrifuging for 10 s at 7550×*g*. The filter tube was then inserted into a clean Eppendorf tube and for the elution of DNA, 200 μL of 72 °C pre-warmed elution buffer (10 mM Tris, pH 8.5) was added to the filter tube and centrifuged for 1 min at 2860×*g*. The eluted DNA was stored at −20 °C for subsequent analysis. DNA was quantified and a volume of the eluate corresponding to 2–3 μg DNA (15–17 μL of eluted DNA) was added to loading buffer (50% glycerol; 2 mM EDTA; 0.4% bromphenol blue), and the DNA solution was run on a 0.8% agarose gel for 60 min at 60 V constant voltage. Gels were stained with ethidium bromide and visualized by ultraviolet light.

2.6. Statistical analysis

P-value<0.05 was considered statistically significant. Prism 6.07 software (GraphPad Software, Inc., San Diego, California) was used for graphs.

The methods are stated in line with STROCSS 2021 guidelines [14].

Table 1
Caspase-3, 8,9 p53, NF-κB and β-actin primer sequences that were used to evaluate gene expression in Real-time PCR.

Primer name	Primer sequence	Product size(bp)
Caspase-3 Forward	5′ AGAACTGGACTGTGGCATTGAG 3′	191
Caspase-3 Reverse	5′ GCTTGTCGGCATACTGTTTCAG 3′	
Caspase-8 Forward	5′ AGAGATGGAGAAGAGGGTCAT 3′	85
Caspase-8 Reverse	5′ CAGCAGGCTCTTGTTGATTTG 3′	
Caspase-9 Forward	5′ CACAGGGTCTGCTGCTCTTTCTC 3′	109
Caspase-9 Reverse	5′ CATTCATCTGTCCCTCTTCCT 3′	
NF-κB Forward	5′ GGAGATCGGGAAAAAGAGC 3′	315
NF-κB Reverse	5′ GACTCCACCATTTTCTTCCTC 3′	
p53 Forward	5′ TCAGTCTACCTCCCGCCATA 3′	322
p53 Reverse	5′ TTACATCTCCCAAACATCCCT 3′	
β-actin Forward	5′ CTGGAACGGTGAAGGTGACA 3′	161
β-actin Reverse	5′ TGGGGTGGCTTTTAGGATGG 3′	

3. Results

3.1. Effects of single, double and triple treatments of 17-AAG, Cap and IR on NF-κB, p53, caspase-3, 8 and −9

The figures (Fig. 1 and 2) show that all treatments could elevate caspase-3, 8 and 9 mRNA expression. Figure 1 shows the results in the bar chart, and Figure 2 uses a heat map and colors to reveal the gene expression. (Both figs by Prism software.). The caspase-3 mRNA expression was significant in 17-AAG and Cap single treatments and 17-AAG/Cap/IR triple treatments. The caspase-8 mRNA expression was significant in 17-AAG and IR single treatments and IR/Cap double treatment. The caspase-9 mRNA expression in 17-AAG and Cap single treatments and IR/Cap double treatment were significant. 17-AAG and Cap single treatments and IR/Cap double treatment down-regulated NF-κB mRNA expression. In Cap and IR single treatments and 17-AAG/IR double treatments, we observed significant p53 down-regulation while upregulation of p53 were significant in a 17-AAG single treatment, Cap/17-AAG and Cap/IR double treatment (Fig. 1 and 2).

3.2. DNA laddering analysis in single, double and triple treatments of 17-AAG, Cap and IR

As shown in Fig. 3, DNA ladder revealed the apoptotic characteristic by forming more oligonucleosomal fragmentation in 17-AAG, Cap and IR single treatments. Whereas, double and triple treatments failed to show obvious oligonucleosomal DNA laddering.

4. Discussion

The aim of this study was to examine the proapoptotic effect of 17-AAG, IR and Cap alone and in double and triple combination in HT-29 human colorectal cancer cell. Proapoptotic effect of 17-AAG by Bcr-Abl depletion, intrinsic pathway initiation, and STAT5 activity increase have been shown [8]. Our result showed that 17-AAG alone can upregulate caspase-3,8,9 and p53 and downregulate NF-κB. Studies have shown that 17-AAG in HT29, HCT116, LoVo, SW480, and SK-CO-1 colon cancer cells increases apoptosis through AKT signaling and NF-kB inhibition and intrinsic apoptotic pathway stimulation [16]. Instrinic pathway of apoptosis is characterized by the release of cytochrome C from mitochondria, leading to the activation of caspases 3. This process is mediated by pro-apoptotic BCL proteins. Increased BCL-2 expression is associated with greater rate of apoptosis in cells. BCL proteins are regulated by cyclin-dependent kinase and p53. Extrinsic apopotitic pathway involves binding of ligand (tumor necrosis factor-related apoptosis-inducing ligand, tumor necrosis factor, Fas ligand) with a death receptor that activates Fas-associated death domain and caspases 8 and 10 to form a death-inducing signalling complex. This leads to the activation of effector caspases [3,6,7] and causes apoptosis [17]. 17-AAG is reported to overcome the resistance of death lignad, tumor necrosis factor-related apoptosis-inducing ligandm, in colon cancer cells [16].

In contrast to our results, 17-AAG had no effects on caspase-3 and -7 at 0.5 μM [15]. Besides, 17-AAG, when combined with oxaliplatin inhibited NF-κB protein [18]. In this study, IR had down-regulate p53 that was in contrast to the result of a study that demonstrated the stimulatory effect of IR on p53. The results of this study also showed resistance to IR in p53-deficient colon cancer cells [19]. This discrepancy may result in cell type differences. As caspase-3 and -9 up-regulation was significant in our results, a study on HCT-116 human colon cancer cells showed a significant increase in caspase-3 and -9 activity [20]. In this study, we observed significant down-regulation of p53 in capecitabine single treatment group that was in line with Garcı'a MA et al. study demonstrated that 5-FU can activate ds-RNA-dependent protein kinase in a p53-independent manner [21]. In the case of double combinations with capecitabine, we observed more NF-κB mRNA

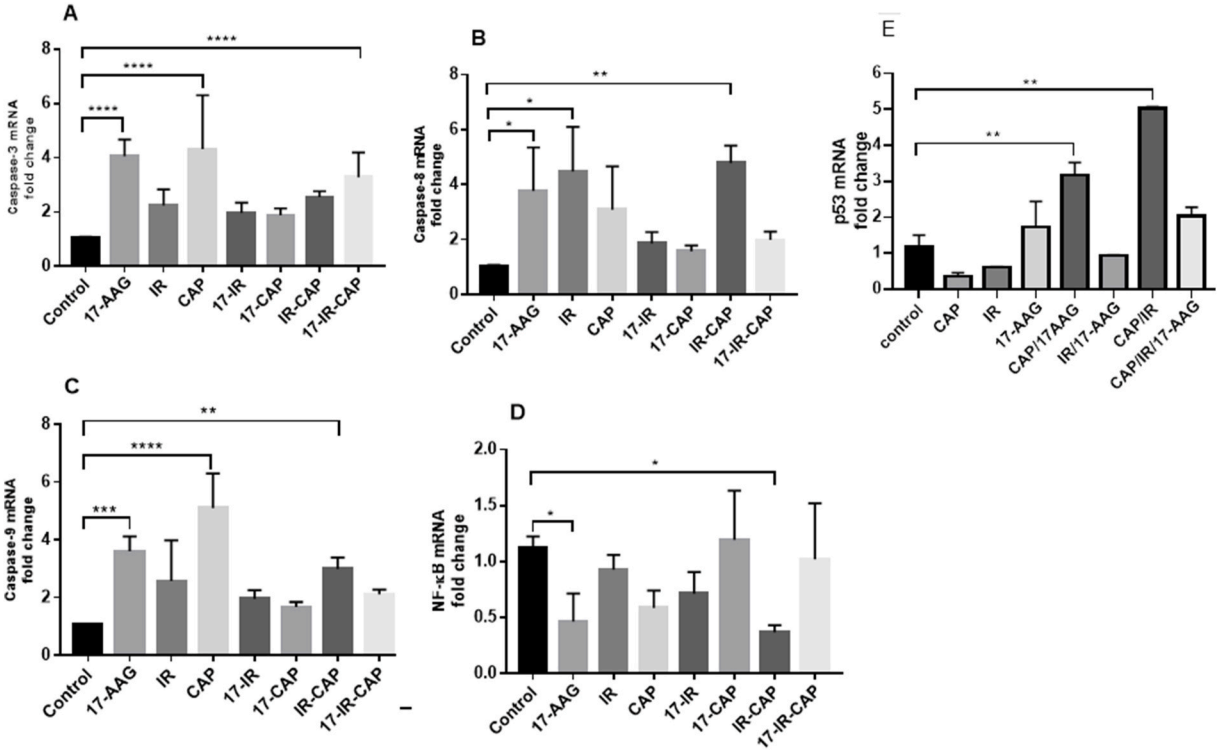

Fig. 1. The effects of capecitabine, irinotecan and 17-AAG alone and in combination on NF-κB, p53, caspase-3,8 and -9 mRNA expression in HT-29 cell line. HT-29 cells were incubated with IC50 values in mono-treatment, 0.5 × IC50 values in double treatment and 0.25 × IC50 in triple treatment for 24 h. The expression level of the caspase-3 (A) and caspase-8 (B) caspase-9 (C) NF-κB (D) and p53 (E) genes compared to β-actin are presented. Data expresses the mean ± SD of three independent experiments. * indicates significantly significant mRNA change of treatments in comparison to control (p-value < 0.05 by one-way ANOVA).

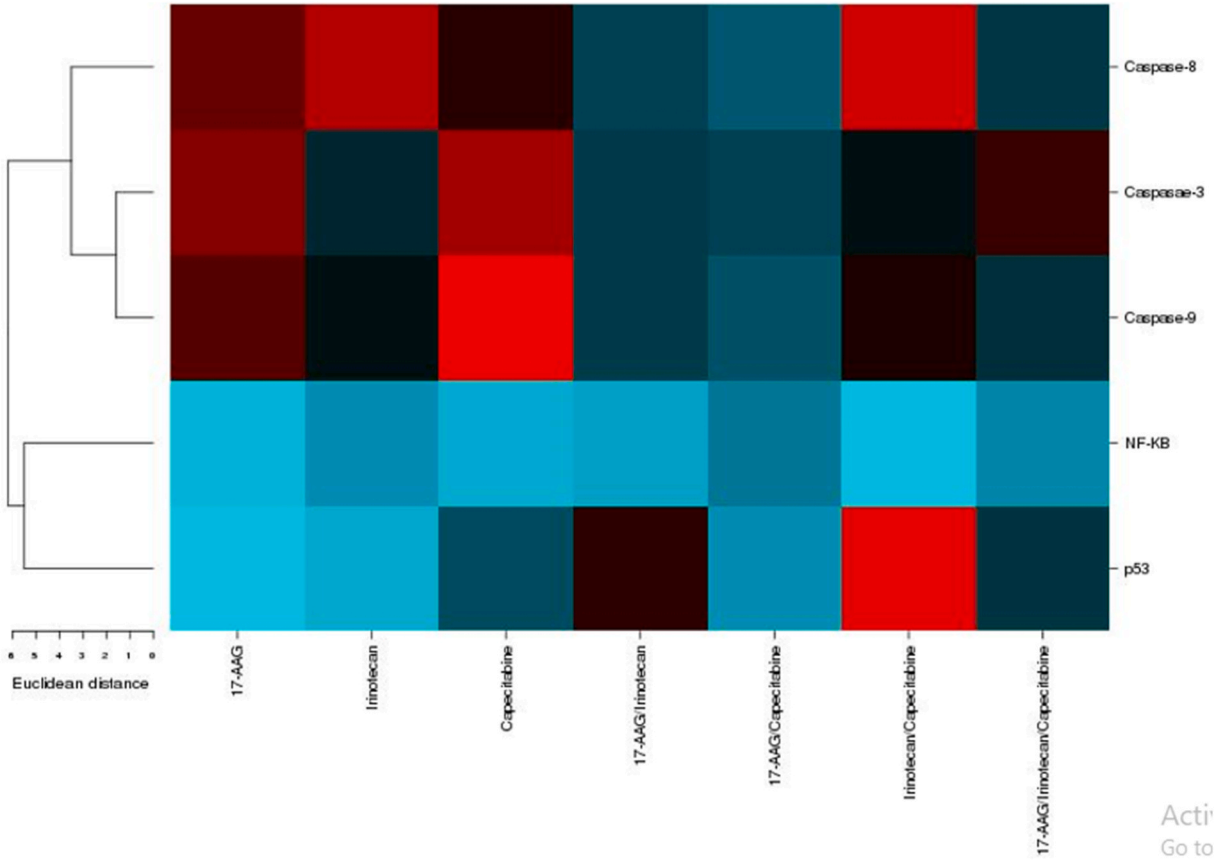

Fig. 2. Clustering of gene expression alterations by using heat map. The heat map reveals changes in the expression of the genes playing crucial roles in cancer apoptosis. The blue indicates downregulated and the red color denotes upregulated genes. This fig draw by Prism software. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

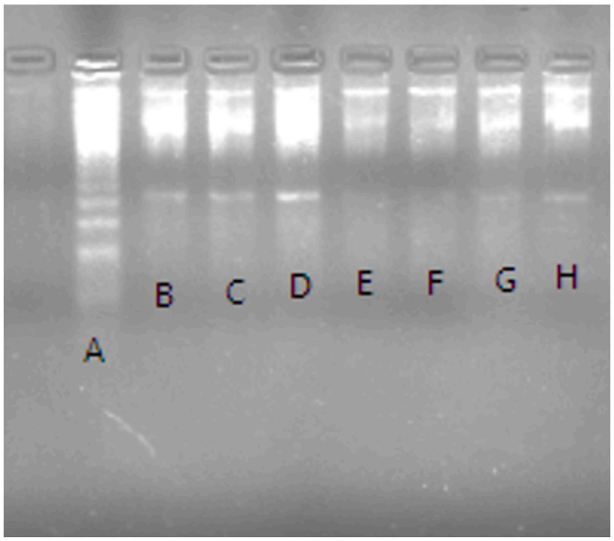

Fig. 3. Gel electrophoresis of DNA from detached HT-29 cells treated with 17-AAG, Cap and IR in single, double and triple treatment groups [2]. positive control, [2] Cap, [2] IR, [2] 17-AAG, [2] Cap/IR, [15] Cap/17-AAG, [15] IR/17-AAG, [2] Cap/IR/17-AAG.

upregulation that was in line with results of a study showed that pre-treatment with capecitabine could sensitize HT29, HCT116, MDA-MB-231 and Panc-28 cells to Turmeric [22]. Our results proposed that IR, when use in double combination, is more effective on caspase-8 and -9 mRNA expression. Results of a study on caspase-3 cleavage after double treatment with 17-AAG and arsenic trioxide showed that 17-AAG in double treatment can activate apoptosis in caspase-3 dependent manner [23]. In accordance to our results, western Blot analysis on HCT-116 cell line showed a significant increase of caspase-3,8 and −9 levels after IR and CG2 (histone deacetylase inhibitor) mono-treatment but this increase was mildly in double combination groups [24]. A study on HT-29 cell line showed that IR in combination with platinum derivative, oxaliplatin, affected cell cycle profile right as well as IR single treatment [25].

Our study is limited to small amount of experimental analysis and only ex-vivo studies are not enough to confirm these findings. Furthermore, Western blot analysis was not performed for protein expression. Our study highlights the importance of multiple therapeutic strategies to increase the effectiveness of chemotherapy, particularly those promoting apoptosis in cancer cells.

5. Conclusion

Overall our findings suggest that 17-AAG could induce apoptosis in a caspase-dependent manner via intrinsic and extrinsic pathway. Nevertheless, in double and triple treatments it seems that 17-AAG could not affect better than mono-treatments on apoptosis pathway which was seen by DNA laddering results. Albeit, to confirm the results of the Real-time PCR, caspase-3,8,9, p53 and NF-κB proteins analysis is suggested. Also, further in-vivo studies are needed to prove the effects of single, double and triple treatments in colorectal cancer treatment and survival.

Provenance and peer review

Not commissioned, externally peer-reviewed.

Ethical approval

All procedures performed in this study involving human participants were in accordance with the ethical ID: (ir.umsu.iec.1396.446) of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Contributors' statement page

Shima Zeynali-Moghaddam: conceptualized and designed the study, drafted the initial manuscript, and reviewed and revised the manuscript.

Dr. Fatemeh Kheradmand : Designed the data collection instruments, collected data, carried out the initial analyses, and reviewed and revised the manuscript.

Dr.Shiva Gholizadeh-Ghaleh Aziz: Coordinated and supervised data collection, and critically reviewed the manuscript for important intellectual content.

Sina Abroon: reviewed revised the manuscript.

Availability of data and material

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Registration of research studies

Urmia University of Medical Sciences, Iran. **(Ethic ID: ir.umsu.iec.1396.446)**:
Unique Identifying number or registration ID: Hyperlink to the registration (must be publicly accessible):

Consent for publication

Not applicable.

Guarantor

Dr. Shiva Gholizadeh-Ghaleh Aziz.

Declaration of competing interest

The authors deny any conflict of interest in any terms or by any means during the study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.amsu.2022.103850.

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