



Original Article



## Correlation of IDH1 gene expression error in breast tumor biopsy in patients with invasive ductal carcinoma

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### Article Info

### Abstract



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One of the most important cancers in terms of worldwide prevalence is breast tumors, which have been less investigated in correlation with the enzyme Isocitrate Dehydrogenase 1 (IDH1) gene. The aim of this study was that expression of this gene could have significant effects on the progression of metastasis and invasive disease in breast cancer patients. We used the molecular method of RT-PCR with SYBR-Green to analyze breast tumor tissue from patients with metastasis and non-metastasis, the latter confirmed by the pathology department of Shohada-e Tajrish Hospital (serving as a control group). Also, patients population and its relationship with the degree of tumor in the IDH1 gene was investigated. The IDH1 gene has shown high expression in patients with metastatic breast cancer rather than in patients with non-metastatic breast cancer. The metastatic samples were compared with non-metastatic samples for IDH1 mRNA expression. In this research work, 72.5% (29 samples) were up-regulated in comparison to 27.5% of samples (11 samples) that did not exhibit high expression (P=0.000). This study examined the IDH1 gene expression, suggesting that changes in this gene's expression could impact the prognosis of breast cancer. However, further research is needed to draw definitive conclusions.

**Keywords:** IDH1 gene, Breast Cancer, Invasive, Correlation.

### 1. Introduction

Breast cancer, a diverse disease with varied clinical outcomes, poses significant challenges to prognosis and treatment decision-making. [1]. Advancements in understanding the molecular pathways responsible for breast cancer have led to new methods for predicting tumor behavior and treatment responses, as well as evaluation of the tumor's aggressive potential [2,3]. Assessing the presence of a tumor in the axillary lymph nodes metastasis in breast cancer patients has a critical approach in the grading of breast cancer as well as the prognosis [4]. A specific diagnostic approach involves identifying metastatic cells by analyzing RNA transcripts from genes, such as CK19 and MGB, which are highly expressed in breast-origin cells but minimally in others. but are found only in small amounts in metastatic non-metastatic tissues. These include

cytokeratin (CK19) and myoglobin (MGB), two indicators that have been studied so far, such assays as an alternative to the use of frozen sections of lymph nodes after surgery, to be examined pathologically have been suggested [5]. In this method, quantification is used by polymerase chain reaction (Real-time PCR) in which the expression of genes such as MUC1, CK19, and MGB is evaluated [6].

Five genes encoding human *IDH* have been identified. In cytosols and peroxisomes, *IDH1*, encoded by the IDH1 gene at 2q33.3, catalyzes oxidative isocitrate decarboxylation (ICT) to 2-ketoglutarate (2KG) (also called  $\alpha$ -ketoglutarate) to remove NADPH from Produce NADP [7]. The enzyme *IDH1* acts as a homodimer and plays an important role in cell defense against oxidative damage, reductive synthesis as a source of NADPH, and regulating the function of dioxygenase enzymes (by producing 2-ke-

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to glutarate as a co-substrate for dioxygenase enzymes) [8,9].

Mutations in IDH1 and IDH2 cause the proteins to gain abnormal functions, converting alpha-ketoglutarate into the cancer-associated metabolite, 2-hydroxyglutarate (2-HG) oncometabolite. Accumulation of 2-HG leads to impaired epigenetic regulation through inhibition of  $\alpha$ KG-dependent histone and DNA demethylases. In addition, Research on small molecule inhibitors targeting mutant IDH1/2 enzymes demonstrates potential in lowering harmful 2-HG levels, offering a promising therapeutic strategy., altering epigenetic regulation disorder, and inducing cell differentiation, have been presented [10].

IDH gene mutations are common in several cancers, including glioma, acute myeloid leukemia, and cholangiocarcinoma, affecting cancer metabolism and treatment responses. Notably, the neomorphic activity of the mutated *IDH* creates distinct patterns in cancer metabolism, epigenetic displacement, and therapeutic resistance [11].

Several pieces of evidence show that during the transformation of epithelial cancer cells, they can acquire mesenchymal properties through a process called epithelial-to-mesenchymal transfer (EMT). This process allows cancer cells to spread by increasing their ability to invade and migrate. Discoveries that mutations in metabolic genes, like FH, SDH, and IDH, trigger the EMT process underscore the link between cancer metabolism and cell migration and invasion [12].

Mutations in isocitrate dehydrogenase (*IDH1*) and *IDH2* are among the most common genetic changes in intrahepatic cholangiocarcinoma (IHCC). *IDH* mutant proteins in intrahepatic cholangiocarcinoma and other malignancies have an abnormal enzymatic activity that allows them to convert alpha-ketoglutarate to 2-hydroxy ketoglutarate, which inhibits the activity of multiple  $\alpha$ KG-dependent dioxygenases and leads to changes in cell differentiation. Mutated *IDH* inhibits hepatic progenitor cell differentiation through 2-HG production and suppression of HNF-4 $\alpha$ , a major regulator of hepatocyte identity and inactivity. These studies provide a functional link between *IDH* mutations, hepatocyte fate, and IHCC pathogenesis [13].

Acute myeloid leukemia (AML), chondrosarcoma, and cholangiocarcinoma are intrahepatic. The mutant protein loses its normal enzymatic activity and acquires a new ability to produce 2-hydroxyglutarate, which is combat to life. 2-HG competitive enzymes Inhibit  $\alpha$ -KG-dependence cells that play an important role in gene regulation and tissue homeostasis. Mutated *IDH* expression disrupts cell differentiation at different cell lines and promotes tumor development in association with other cancer genes [14].

Specific mutations in the isocitrate dehydrogenase (*IDH*) gene were detected in several gliomas, including polymorphic oligodendroglioma and glioblastoma, as well as in leukemia. These mutations produce alpha-ketoglutarate, and 2-hydroxy ketoglutarate (2-HG) distinctly. 2-HG accumulates in very high concentrations, which inhibits the function of alpha-ketoglutarate-dependent enzymes [15]. This phenomenon leads to hypermethylation of DNA and histones and as a result, changes the expression of genes that can activate oncogenes and inactivate tumor suppressor genes [16].

The study of *IDH1* gene expression by quantitative assay was performed to achieve the hypothesis that changes

in the expression of this gene could have significant effects on the progression of metastasis and invasive disease in breast cancer patients.

## 2. Materials and methods

We collected 40 invasive ductal carcinoma samples with metastatic symptoms and 40 non-metastatic breast tumor biopsies from Shohada Tajrish Hospital (Tehran, Iran) in June 2021. Data for all patients were saved for analysis. The diagnosis of all breast cancer tumors was confirmed by immunohistochemistry (IHC) staining in the pathology department.

In the classification of samples, two groups of metastatic tumors and non-metastatic tumor samples were considered. The beta-actin gene was used as an internal control for the accuracy of the experiment. Twenty breast tumor specimens that tested negative for metastasis by pathology tests were considered controls. Forty samples of breast cancer tissue with metastasis were studied as a study group in this project.

Tumor severity according to the international standard AJCC-02-TNM was previously classified by the pathologist into groups 1 = 1, 2 = 2a, 3 = 2b, 4 = 3a, 5 = 3b, 6 = 3c, 7 = 4, which in There were Stage 3 and 4 samples for metastatic cases and Stage 1 and 2 for non-metastatic cases, and the grade of the tumor was divided by the pathologist into three categories: I, II, and III, and cases without metastasis were mostly identified with grade 1. In addition to the expression and non-expression of genes, the expression of these genes was also examined by increasing and decreasing the degree and severity of the tumor. The mean age selected was 50 years in the study of tumor samples that did not have tumor metastases.

### 2.1. Total RNA isolation and cDNA synthesis

RNA was isolated from invasive ductal carcinoma samples by homogenization in 1 mL of Tripure reagent, using approximately 50  $\mu$ g of each breast tumor specimen. Following homogenization, the mixture was incubated at room temperature for 5 minutes, then 0.2 mL of chloroform was added, mixed thoroughly, and centrifuged at 12,000 x g for 15 minutes at 4°C. RNA was precipitated retained and added isopropyl alcohol. The upper aqueous phase was transferred to a new tube and centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA pellet was then washed twice with 1 mL of 70% ethanol. The RNA pellet was resuspended in DEPC-treated water to achieve a final concentration of 0.5  $\mu$ g/ $\mu$ L.

### 2.2. Reverse transcription and Real-time PCR

Reverse transcription, 5  $\mu$ g of total RNA was mixed with 0.5  $\mu$ g of oligo(dT) and 16  $\mu$ L of RNase-free water, then incubated at 64°C for 10 minutes. Total RNA extracted. The primers for SYBR Green real-time PCR were designed specifically for the *IDH1* gene and for the ACTB gene ( $\beta$ -actin) as an internal control, Table 1. The assays were repeated in their entirety for each measurement.

Reverse Transcription is carried out with the Superscript First-Strand Synthesis System for RT-PCR. The following procedure is based on Invitrogen's protocol (total RNA 5 g, random hexamers (50 ng/ $\mu$ l) 3  $\mu$ l, 10 mM dNTP mix 1  $\mu$ l, DEPC H<sub>2</sub>O to 10  $\mu$ l). Incubate the samples at 65°C for 5 min and then on ice for nearly 1-1.5 min. Prepare reaction master mixture. For each reaction (10x RT

**Table 1.** Primer design with gene runner and NCBI BLAST for B-Actin and *IDH1* genes.

Genes	Forward primer 1	Reverse primer 1	product length
Homo sapiens <i>IDH1</i> , mRNA			
NCBI Reference Sequence: <a href="https://www.ncbi.nlm.nih.gov/nuccore/NM_001282387.1">NM_001282387.1</a>	TAGGTCGTCATGCTTATGGGG	CACCACCACCTTCTTCAAAG	144
Homo sapiens actin beta ( <i>ACTB</i> ), mRNA			
NCBI Reference Sequence: <a href="https://www.ncbi.nlm.nih.gov/nuccore/NM_001101.5">NM_001101.5</a>	GAGAAGATGACCCAGATC	CACGATGCCAGTGGTACGG	108

buffer 2  $\mu$ l, 25 mM MgCl<sub>2</sub> 4  $\mu$ l, 0.1 M DTT 2  $\mu$ l, RNAase outing 1  $\mu$ l). Add the mixture to the RNA/primer and then place at room temperature for 3 min. Add 1  $\mu$ l (50 units) of Superscript II RT to each tube, mix, and incubate at 25°C for 10 min. Incubate the tubes at 42°C for 50 min, heat inactivates at 70°C for 15 min and then chill on ice. Add 1  $\mu$ l RNase H and incubate at 37°C for 20 min. Store the 1st strand cDNA at -20°C until use for Real-time PCR.

The protocol and design of the study have been approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences with the reference code of IR.SBMU.RETECH.REC.1401.636. Written and signed consent was obtained from each patient or their first-degree relatives after comprehensive explanation was given about the aims and procedures of the study.

### 3. Results

Breast cancer is a heterogeneous disease that has different pathological and cytological features. The cytogenetic and molecular diagnosis is vital for the prognosis and treatment of this cancer. The application of molecular markers such as *IDH1* Gene made the prognosis much more accurate which can predict the metastasis potential [17]. Regarding different studies, based on microarray analysis on breast cancer samples, they found that several genes showed up-regulation.

#### 3.1. RT-PCR results

RT-PCR was conducted on the tumor samples using the protocol described previously. Data analysis was performed using REST and SPSS version 20, incorporating t-tests and Pearson's chi-square tests.

#### 3.2. REST Analyses

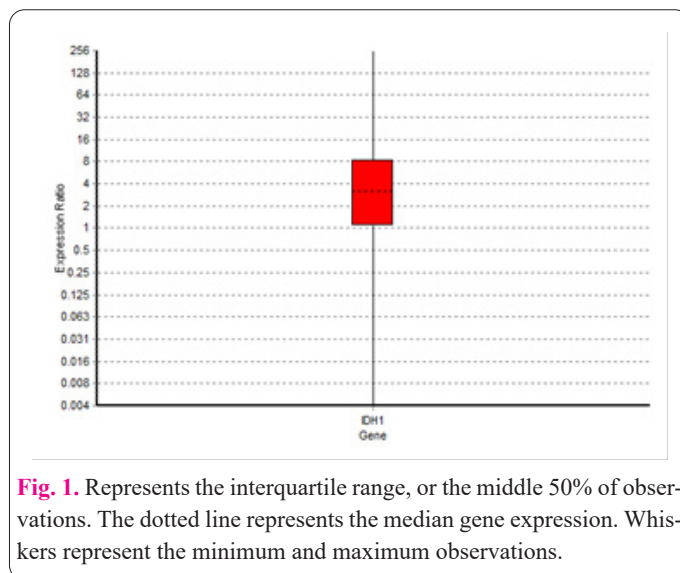
*IDH1* expression was significantly upregulated in the tumor group compared to the control group, with a mean fold change of 2.711 (standard error = 0.542;  $P < 0.001$ ). Figure 3. Tables 2 and 3. Also, the graph of relative expression for the *IDH1* gene is shown in Figure 3a related to the upregulation of *IDH1* in metastatic samples. Also, Figure 1 represents the interquartile range or the middle 50% of observations. The dotted line represents the median gene

**Table 2.** Relative expression report.

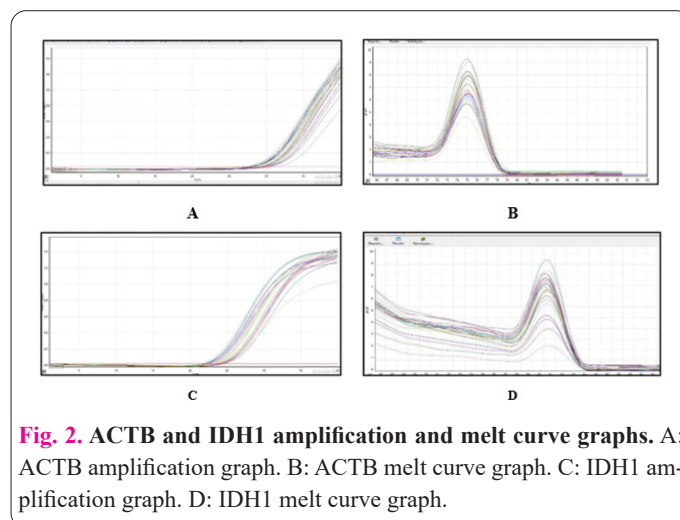
Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
<i>IDH1</i>	TRG	0.9112	2.884	0.542 - 14.701	0.064 - 99.492	0.000	UP
<i>ACTB</i>	REF	0.8664	1.000				

$P(H1)$  - Probability of alternate hypothesis that difference between sample and control groups is due only to chance.

*IDH1* is UP-regulated in the sample group (in comparison to the control group) by a mean factor of 2.884 (S.E. range is 0.542 - 14.701). The *IDH1* sample group is different from to control group.  $P(H1) = 0.000$ , TRG – Target. REF – Reference.



**Fig. 1.** Represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.



**Fig. 2.** *ACTB* and *IDH1* amplification and melt curve graphs. A: *ACTB* amplification graph. B: *ACTB* melt curve graph. C: *IDH1* amplification graph. D: *IDH1* melt curve graph.

expression. *IDH1* and *ACTB* amplification graph as Figure 2 A-D.

#### 3.3. Statistical Analyses

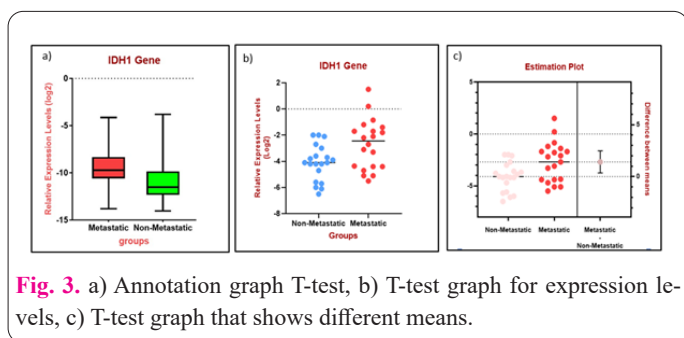
Using SPSS for analysis, we compared *IDH1* mRNA expression between metastatic and non-metastatic samples. This analysis revealed that 72.5% of the samples

**Table 3.** Fold change report.

	FC Metastatic samples	FC Non-Metastatic samples
Number of values	40	40
25% Percentile	0.0006250	0.0002000
Median	0.001200	0.0003000
75% Percentile	0.003125	0.001075
Mean	0.004260	0.002675
Std. Deviation	0.01003	0.01129
Std. Error of Mean	0.001586	0.001785

**Table 4.** SPSS assessment.

Exp. <i>IDH1</i>					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	negative	11	27.5	27.5	27.5
	positive	29	72.5	72.5	100.0
	Total	40	100.0	100.0	



**Fig. 3.** a) Annotation graph T-test, b) T-test graph for expression levels, c) T-test graph that shows different means.

(29 out of 40) exhibited upregulation of IDH1, whereas 27.5% (11 out of 40) did not show high expression, with a standard deviation of 0.542 (Table 4).

### 3.4. Graph Pad Prism Analyses and T-test Graphs

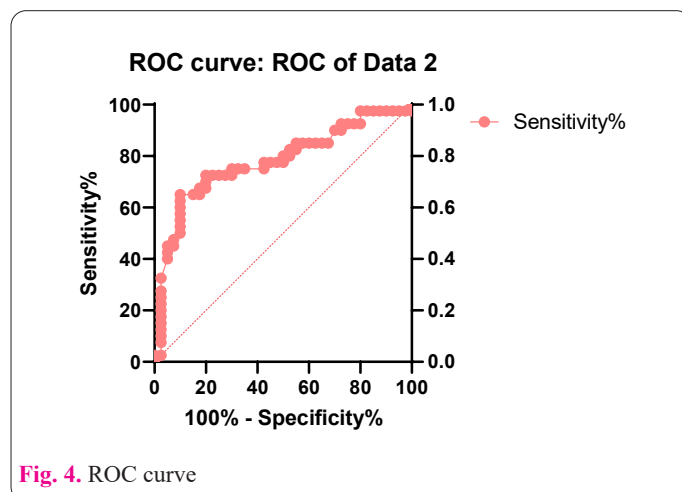
Analysis using GraphPad Prism revealed a statistically significant difference in IDH1 gene expression between tumor and normal samples ( $P < 0.001$ ), Figure 3b.

In examining the relationship between *IDH1* gene expression in metastatic cancer samples, nearly 50% (20 samples) were in grade 2 and 50% (20 samples) were in grade 3, of which 13 grade 2 samples showed an increase in *IDH1* gene expression (65%), and 17 grade 3 samples also showed upregulation for this gene, in other words, 32.5% of the upregulated items were in grade 2 and 42.5% of the upregulated samples belonged grade 3 groups. About Stage, 50% of the samples (10 samples) were in Stage 2, and 95% of the samples (19 samples) were in Stage 3, of which 25% of the Upregulated samples were in Stage 2 and 47.5% of the total upregulated genes related to stage 3. A significant association was found between tumor grade and IDH1 expression ( $P = 0.032$ ), indicating that IDH1 expression tends to increase with higher tumor grades. Also, in examining the significance of the relationship between stage increase in patients with increased expression of the *IDH1* gene by Pearson Chi-square test, a significant relationship was found ( $P = 0.000$ ) (Table 5) and The ROC curve analysis demonstrated high specificity and sensitivity of IDH1 expression in distinguishing between metastatic and non-metastatic samples, as illustrated in Figure 4

### 4. Discussion

IDH1, an enzyme integral to the Krebs cycle, catalyzes the conversion of isocitrate to alpha-ketoglutarate, playing a vital role in cellular metabolism. Therefore, paying attention to this enzyme and its gene can help us understand cell function. It is not far-fetched that the study of this gene in cancer patients could be very useful in terms of its high prevalence, the costs imposed on patients and many other cases, and the close relationship between cancer and cell metabolism. In this regard, studies have been conducted by researchers on this enzyme and its genes [18].

For example, a study by Lenny Dang et al found that different mutations in the *IDH1* enzyme gene are a common feature of early human brain cancers. These mutations occur in an amino acid residue at the active site of the enzyme *IDH1* and convert arginine 132 to histidine, which changes the activity of the enzyme instead of producing alpha-ketoglutarate from isocitrate, producing 2HG (2-hydroxyglutarate). It is made from isocitrate. The production of 2-HG, which is known as an oncometabolic, in this type of mutation causes the increase and accumulation of this substance in the body, which in turn contributes to the formation and malignant development of glioma [19]. A similar study on glioma was conducted by Hao Chen et al., Which shows that the 2HG product produced by the



**Fig. 4.** ROC curve

mutant *IDH1* may increase neural activity by mimicking the activity of glutamate on the NMDA receptor, leading to gliomas that *IDH1* is mutated in them, causing more seizures in patients [20].

Most studies on the *IDH1* gene have been linked to gliomas, and fewer studies have been performed on other cancers. The association of this gene with other cancers can be referred to the study of Jingyi Wu et al. High expression of *IDH1* is associated with high tumor grade [21]. For example, a study by Bingdi Yan et al found that mutations in *IDH1* increased NSCLC cell migration and proliferation [22].

Despite its global prevalence, breast cancer's association with the IDH1 gene remains underexplored. One of the most important studies in this field has been done by Wen-Shan Liu et al. In this study, it was found that the expression of *IDH1* gene in breast cancer tissue shows a decrease in expression. In this study, it was concluded that the enzyme *IDH1* produces the NADPH product by converting isocitrate to alpha-ketoglutarate, which is a major reducing agent for glutathione regeneration (GSH) by GSH reductase and the NADPH-dependent thioredoxin system, both of which protect cells are important against oxidative damage. *IDH1* can produce cytosolic NADPH in cells, indicating that they defend against oxidative stress induced by reactive oxygen species. Thus, disruption of the *IDH1* enzyme and gene can upset the redox balance by reducing the production of NADPH, and the disorder benefits the cancer cell. Also in this study, it was found that reducing the expression of IDH1 increases the level of HIF1 alpha. HIF1 alpha can increase cancer cell metastasis through EMT [23].

On the other hand, the *IDH1* enzyme also helps cell proliferation and growth by promoting metabolism. Due to this, cancer cells can not only suppress the expression of the *IDH1* gene in the early stages of the tumor and before metastasis by reducing NADPH production and redox imbalance but also by increasing the level of HIF1 alpha in tumor metastasis, in later stages. Disease and metastatic progression also increase cellular metabolism by producing *IDH1* gene expression to produce energy for themselves in tumor growth and development [24].

Our study found that IDH1 gene expression levels were significantly higher in patients with metastatic breast cancer and invasive ductal carcinoma compared to those without metastatic features. The results of this experiment also showed that by increasing the degree and stage of tumors, the expression of the *IDH1* gene also increased in more related samples. According to the obtained results, it seems that in the early stages of the tumor, tumor cells are able to suppress the expression of the *IDH1* gene and through this can reduce the redox balance, and also increase the level of HIF1 alpha to spread to other areas (metastasis) to they help their growth and development, but as cancer progresses, high expression of *IDH1* can increase fuel supply to cancer cells by increasing metabolism and acting to progress cancer. Therefore, tracking the increase in the expression of this gene can be used to understand the progression and prognosis of the disease. However, bioinformatics in this issue may be considered [25].

## 5. Conclusion

*IDH1* gene has shown high expression in patients with metastatic breast cancer rather than in patients with non-

metastatic breast cancer. Hence, the observed alterations in IDH1 expression hold potential for future metastasis prediction studies, both in larger cohorts and across different cancer types.

## Competing interests

The ethics code is <http://ethics.research.ac.ir/SBMU.RETECH.REC.1401.636> in the medical research committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

## Conflict of interest statement

The authors declare no conflict of interest.

## Consent for publications

The author read and approved the final manuscript for publication.

## Ethics approval and consent to participate

No human or animals were used in the present research.

## Informed consent

The authors declare not used any patients in this research.

## Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request

## Authors' contributions

Behzad Rostami, Muzi Chen, Neda Mansouri and Abolfazl Movafagh designed the study and performed the experiments, Neda Mansouri, Aliasghar Keramatinia, Abdol Rahim Nikzamir and Saeed Karima collected the data, Rezvan Ghadyani and Seyed Jalil Hoseini analyzed the data, Behzad Rostami, Muzi Chen, Batool Ghorbani Yekta, Sepehr Kahrizi and Abolfazl Movafagh prepared the manuscript. All authors read and approved the final manuscript.

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