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MicroRNA Upregultion in MKN-45 Gastric Cancer Cell Line by Ibuprofen Treatment: Highlighting the Importance of miR-22

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ABSTRACT

Nonstroidal anti-inflammatory drugs (NSAIDs) including ibuprofen have been shown to decrease the growth of cancer cells. Owning to the involvement of microRNAs in development of cancer, the aim of this study was to evaluate the effect of ibuprofen on the expression level of some microRNAs including hsa-miR-4290, hsa-miR-22 and hsa-Let-7b in MKN-45 cells. First, the growth inhibitory effect of ibuprofen on MKN-45 was evaluated using MTT and trypan blue exclusion assays. Second, the gene expression level of micoRNAs was evaluated using real-time PCR. Time- and dose-dependent proliferation reduction was seen in MKN-45 gastric cancer cells treated with ibuprofen. Results showed that ibuprofen treatment increased the expression level of miR-4290, miR-22 and Let-7 by 2.14, 57.8 and 1.58 folds, respectively, relative to control MKN-45 cells. Results clearly highlighted the fact that miR-22 plays a critical role in growth inhibition of ibuprofen treated MKN-45 gastric cancer cells. Therefore, we concluded that overexpression of these microRNAs in ibuprofen-treated MKN-45 clearly indicated the tumor suppressor role of them in gastric cancer prevention.

Keywords: Nonstroidal anti-inflammatory drugs, Ibuprofen, MKN-45 cell line, MicroRNAs

INTRODUCTION

Gastric cancer derived from the lining of stomach is considered as one of the leading causes of death in worldwide [1,2]. Adenocarcinoma of the stomach is assigned as the most common type of gastric cancer. The etiology of stomach adenocarcinoma is associated with chronic inflammation, exposure to carcinogens in dietary and genetic susceptibility [3,4]. Regardless of the several causes of gastric cancer, the molecular mechanism of carcinogenesis is still unclear. Therefore, the diagnosis and also the treatment of stomach are generally poor. In this line, illustration of the main factors including microRNAs and/or signaling elements involved in carcinogenesis of gastric cells and/or in response to effective drugs seems to have shed light on the several aspects of carcinogenesis as well as early detection, prevention of cancer development and medical intervention [4].

MiRNAs are a class of noncoding RNAs by a length of 18-25 nucleotides which are involved in a variety of physiological processes including cell growth, proliferation and apoptosis by induction of RNA degradation and/or prevention of mRNA translation [5-9]. In general, microRNAs are classified to oncogenes and tumor suppressors through repressing the expression of tumor suppressor genes and oncogenes, respectively [10]. In this line, the involvement of miRNAs in several aspects of cancer development has been reported and characterized in the number of studies. For example, the aberrant expression of miR-301a, miR-21, miR-145 and miR-610 have been found in the pivotal phases of gastric cancer including promotion, invasion and metastasis [11-13]. In addition, recent studies have been shown that plasma miRNA-199a-3p levels could be considered as a promising diagnostic biomarker for early gastric cancer [14]. On the other hand, upregulation of miR-200c and downregulation of miR-21 have been elucidated to enhance chemosensitivity to cisplatin in gastric cancer [15,16]. It has been reported that the growth of gastric cancer is suppressed by miR-4290 *in vitro*. In addition, the downregulated expression of miR-29c and miR-22 and Let-7 have been shown in gastric cancer [12,17]. Together, detection of microRNAs in several

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stages of gastric cancer and/or during the action of drugs seems to be applied as a promising therapeutic target in gastric cancer [18].

Nonstreroidal anti-inflammatory drugs (NSAID) are commonly used to suppress the activity of cyclooxygenase enzymes (COX1 and COX2) as well as the synthesis of eicosanoid hormones, especially prostaglandins with the aim of alleviation of pain and inflammation [19]. However, the antiproliferative effect of NSAIDs has been reported in several types of cancer at extra doses and/or long term disposal [20,21]. Ibuprofen has been reported to induce cell death in HT-29 human colorectal cancer cells, MKN-45 gastric adenocarcinoma cells, DU-145 prostate cancer cell line and human cutaneous cell line [19,22-24]. Inhibition of proliferation, migration and angiogenesis are considered as the main causes of ibuprofen in cancer prevention [22,24]. It has been suggested that NSAID-induced apoptosis mediated by a number of molecular mechanisms. However, the molecular mechanism of NSAID in cancer prevention has not completely been elucidated yet. It has been initially proposed that NSAIDs induces apoptosis mediated by inhibition of cyclooxygenases. Then, COX-independent mechanisms have been clarified in several studies [19,25,26]. In this line, the involvement of a number of signaling factors including HIF, cyclins, NF-κB, β-catenin and GSK3β have been found in ibuprofen-induced cell death in cancer cells [26-28].

In this study, we aimed to investigate the antineoplastic effect of ibuprofen on the expression levels of miR-22, miR-4290 and Let-7 in MKN-45 human gastric adenocarcinoma cell line. Increased level of miRNAs of 22, 4290 and Let-7 in ibuprofen-treated MKN-45 clearly indicated the tumor suppressor role of them in gastric cancer prevention.

MATERIALS AND METHODS

Materials

Dimethyl sulfoxide (DMSO) and Ethylen ediaminete traacetic acid (EDTA) were supplied by Merk (Germany). RNA extraction kit was purchased from Bioflux (China) and cDNA synthesis kit was obtained from Quiagene (Iran). The cell culture medium (DMEM) was purchased from Gibco (Germany). Neutral red, trypan blue, penicillin, streptomycin, amphotericin B, fetal bovine serum (FBS), trypsin and Ibuprofen were purchased from Sigma (Germany).

Cell Culture and Treatment

MKN-45 cell line was obtained from National Cell bank of Iran (NCBI) affiliated to Pasteur Institute of Iran. MKN-45 cells were cultured in DMEM/F12 containing 10% (v/v) heat-inactivated FBS in the presence of 100 units/mL penicillin and 100 μg ml^{-1} streptomycin. MKN-45 cells were incubated at 37 °C in a humidified incubator under an atmosphere of 95% air and 5% CO_2. Cell culture media were changed twice per week. Ibuprofen stock solution was prepared in DMSO at 200 mM concentration and aliquots were kept at -20 ºC. In test, after 48 h seeding of cells in plate, cell medium was removed and replaced with fresh medium containing 3% FBS and various concentrations of ibuprofen in triplicates for 24 and 48 h. Both untreated MKN-45 cells and MKN-45 cells treated with DMSO (1% v/v) were used as controls.

MTT Assay

To analysis the ability of ibuprofen to inhibit cell growth, MKN-45 cells were cultured in 96-well plates (5000 cells/well). The cells were allowed to attach and grow for 24 h in culture medium with 2% FBS. Next, the cells were treated with various concentrations of Ibuprofen. After 24 and 48 h, MTT assay was performed by adding 20 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO USA) to cells and incubated for 3 h at 37oC, 5% CO_2. Then media was removed and 100 μl of dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO USA) was added to each well to dissolve the formazan crystals. Absorbance at 545 nm with background subtraction of 630 nm was measured with an ELISA plate reader. Percentage of cell growth was determined relative to control. All experiments were done in triplicate.

Trypan Blue Assay

The cytotoxic effect of Ibuprofen on the viability of MKN-45 cells was evaluated by Trypan blue exclusion test. In brief, MKN-45 cells were treated with different doses of ibuprofen and the cells were stained by trypan blue after interval times. Then, viable cells as the white cells and died

cells as blue cells are counted using a hemocytometer.

RNA Extraction

Total RNA was isolated from cultured cells using Tripure isolation reagent (Roche Diagnostics, Germany) according to the manufacturer's instructions. Briefly, 500 µl of Tripure solution was added to 150 µl of cell suspension. After lysing of cell, 200 µl of chloroform was added, was shaken with hand and centrifuged at 12000 g and 4 ºC for 15 min. Then, isopropanol (500 µl) was added to upper phase of solution and was kept for 10 min at room temperature. After 15 min centrifuging of solution at 12000 g and 4 ºC, the upper phase was removed and precipitate was eluted with 75% ethanol. Then, ethanol was removed and dried precipitate was dissolved in DEPC-treated RNase free water. The accuracy of the method was evaluated by 1% gel agarose electrophoresis staining with ethidium bromide.

Primer Designs for microRNAs (miRNAs)

We obtained the miRNA sequences from http://www.mirbase.org (Table 1). In this study, we used the stem-loop method to design specific primers for the miRNAs. Specific stem-loop RT primers comprised 44 nt of the stem-loop sequence according to Chen *et al*., with the six 3′nt of the desired mature miRNAs.23 Forward primers included the first 12 nt from the 5′end of the mature miRNA sequences and a 6 additional nt at 5′end of the primers to obtain the Tm to 60 ± 1 °C. A universal primer with 16 nt of the stem-loop sequence in RT primers was used as a reverse primer (Table 2).

Quantitative Real-time PCR

The RNA reverse transcription reaction was carried out with the QuantiTect® Reverse Transcription kit (Qiagen) according to the manufacturer's protocol. In brief, Total RNA was incubated in the presence of RT-primers at 65 ºC for 5 min and immediately was placed on ice. Then, a mixture including AMV Reverse Transcriptase, buffer, dNTPs and RNase inhibitor were added and reversed transcribed at 42 ºC for 60 min and then, reaction was stopped at 70 ºC for 5 min. Quantitative real-time polymerase chain reaction (qRT-qPCR) was performed with an instrument (Qiagen, Germany) a Rotor-Gene 3000 System (Corbett Research, Australia) using SYBR premix Ex Taq technology (Takara Bio Inc., Otsu, Japan). The SYBR Green master mix (2X) (10 µl) was added to 0.5 µl of the complementary DNA sample, 0.5 µl of forward and reverse primers (10 pmol) and 9 µl of nuclease-free water to conduct PCR in 20 µl of reaction mixture. Thermal cycling conditions involved an initial activation step for 4 min at 94 °C followed by 40 cycles, including a denaturation step for 10 s at 95 °C and 30 cycles of annealing/extension step for 10 s at 60 °C. Melting curves were analyzed to validate a single PCR product of each primer. Comparative quantitation analysis ($2^{-\Delta\Delta Ct}$ method) was used to determine fold change in gene expression and normalized to the housekeeping gene, GAPDH, which has been validated as an endogenous control gene for current studies [29].

Target Prediction of miRNA

To analyze potential microRNA targets, the microRNA databases and target prediction tools miRBase (http://microrna.sanger.ac.uk/), miRwalk (http://mirwalk.uni-hd.de/), miRANDA (www.microrna.org/) and TargetScan (http://www.targetscan.org/index.html) were used.

Statistical Analysis

All *in vitro* assays were repeated in three independent experiments. The data were analyzed using GraphPad Prism (Graph Pad Software, version 6, San Diego California, CA, USA). Homogeneity of variance was first tested, and the significance of difference between multiple groups was evaluated by one-way ANOVA, followed by Bonferroni post-hoc test. $p < 0.05$ is considered statistically significant. Each point or column represents the mean ± SEM (n = 3). *P < 0.05, ** P < 0.01, ***P < 0.001, ****P < 0.0001.

RESULTS

Effect of Ibuprofen Treatment on Viability of MKN-45 Cells

To evaluate the viability of gastric MKN-45 cells exposed to Ibuprofen, trypan blue and MTT assays were used. For this aim, MKN-45 cells were treated with different doses of Ibuprofen (100, 200, 300, 500, 800 and 1000 µM). After 24 h and 48 h treatment, treated and untreated MKN-45 cells were assayed by these two

Table 1. MicroRNA (miRNA) Sequences

miRNA length	miRNA sequence	miRNA
22 nt	CUAUACAACCUACUGCCUUCCC	Hsa-let-7b
22 nt	AAGCUGCCAGUUGAAGAACUGU	Hsa-mir-22
19 nt	UGCCCUCCUUUCUUCCCUC	Hsa-mir-4290

Table 2. The Sequences of Stem-loop RT, Forward and Reverse Primers and PCR Product Lengths

Gene ID	Primer/product length (bp)	Sequence (5ˈto 3ˈ)
Hsa-let-7b	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGAAG
	Forward primer	CGCGCTATACAACCTACTGCCT
	Reverse primer	GTGCAGGGTCCGAGGT
	Product Length	70
Hsa-mir-22	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGTT
	Forward primer	ACGCAAGCTGCCAGTTGAAG
	Reverse primer	GTGCAGGGTCCGAGGT
	Product Length	70
Hsa-mir-4290	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAGGGA
	Forward primer	CGCTGTATCAGGGAGTCAGG
	Reverse primer	GTGCAGGGTCCGAGGT
	Product Length	69
GAPDH	RT primer	Random hexamer
	Forward primer	ACTCTGGTAAAGTGGATATTGTTGC
	Reverse primer	GGAAGATGGTGATGGGATTTC
	Product Length	162

methods. On the basis of obtained results, after 24 h and 48 h of incubation, the Ibuprofen significantly reduced the viability of MKN-45 cells at different concentrations compared with the control group, and these effects were stronger as dose and time increased. The viability of MKN-45 cells exposed to 1% DMSO was nearly the same as untreated control MKN-45 cells and was about 90%. The concentrations of Ibuprofen producing half maximum proliferation inhibition (IC_{50}) were about 700 μM and 500 μM, after 24 h and 48 h treatment, respectively, compared to control cells and/or cells (Fig. 1). The results clearly indicated that ibuprofen reduced the viability of MKN-45 cells in a time and dose-dependent manner.

Effect of Ibuprofen on the Expression Level of Let-7, miR-22 and miR-4290

Given that microRNAs play the important role in development of gastric cancer, we examined whether ibuprofen is able to affect the gene expression levels of Let-7, miR-22 and miR-4290 which seem to be down-regulated in gastric cancer. In this line, MKN-45 cells were treated with 500 μM of Ibuprofen and the gene expression level of micoRNAs was evaluated using real-time PCR. GAPDH was considered as control gene. To confirm the accuracy of extraction and reverse transcription of RNA, electrophoresis was performed. Figures 2A and B showed two bands of 18S and 28S of RNA and the 70 bp fragments of amplified

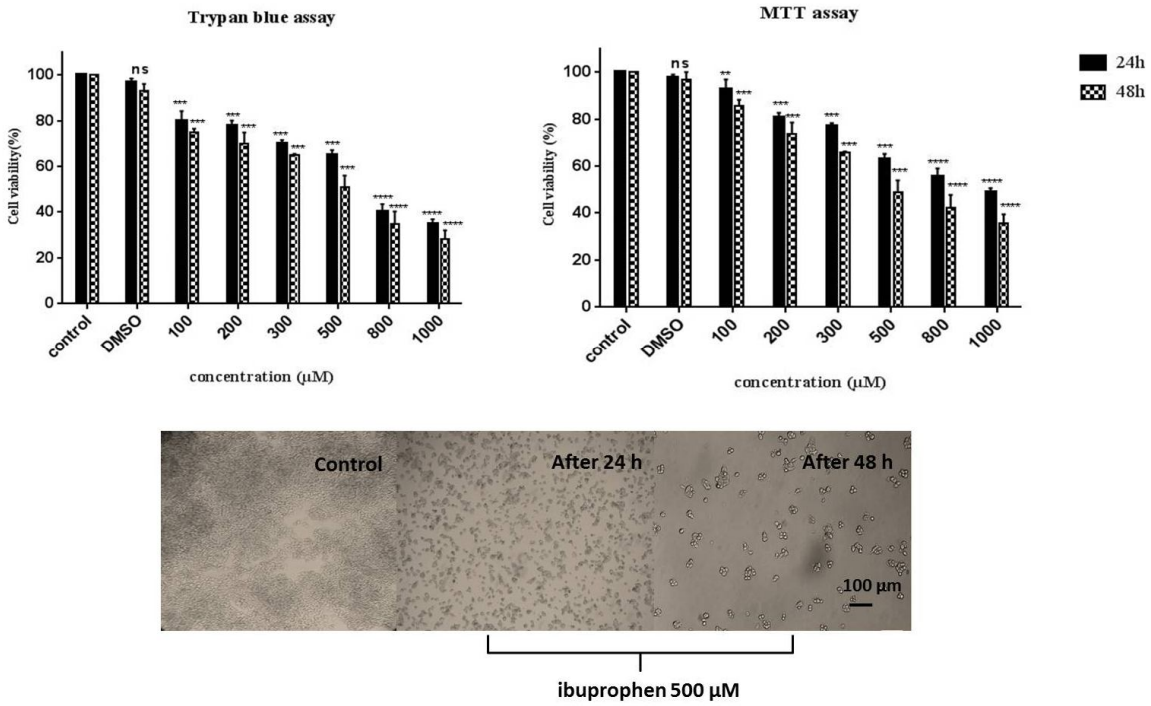

Fig. 1. Effect of Ibuprofen treatment on viability of MKN-45 cells. MKN-45 cells were treated with different doses of ibuprophen (100-1000 µM) and the viability of cells was determined via trypan blue staining after 24 h and 48 h treatment. Each column in the graph was the mean ± SEM of three independent experiments. (ns = non-significant, *P < 0.05, **p < 0.01, ***p < 0. 001, ****p < 0. 0001) (GraphPad Prism 6 software).

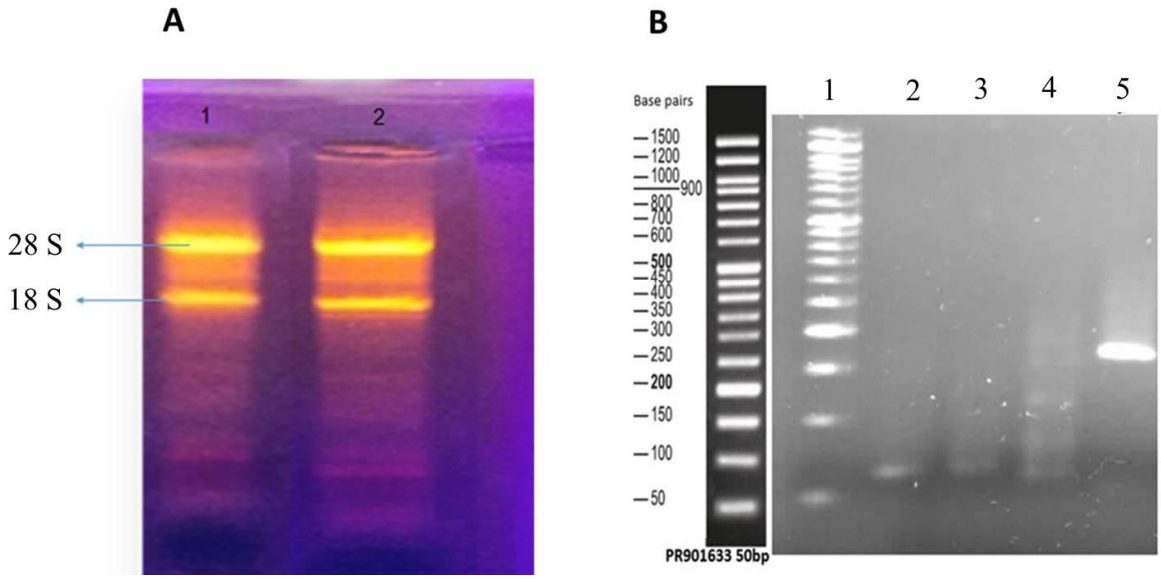

Fig. 2. Electrophoresis of agarose gel. The RNA related to ibuprophen-untreated (lane1) and -treated MKN-45 cells (lane 2) with 500 µM ibuprophen was extracted and electrophoresed (A). Then, cDNA was synthesized using specific primers and fragments by 70 bp length was electrophoresed (B). 1, Ladder; 2, hsa_mir_Let7b; 3, hsa_mir_4290; 4, hsa_mir_22; 5, GAPDH (162 bp).

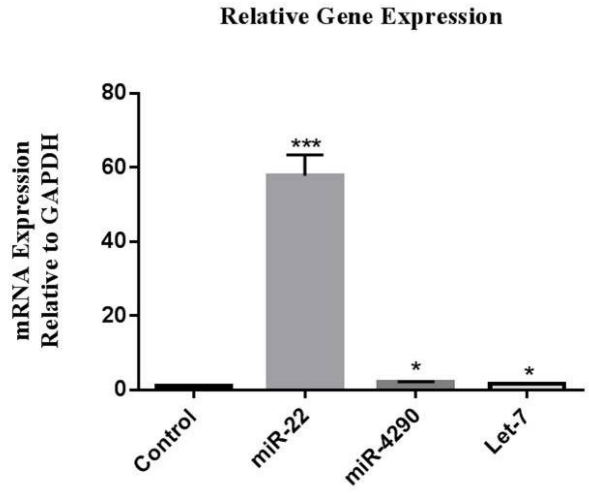

Fig. 3. Effect of ibuprophen on the expression levels of miRNAs. MKN-45 cells were exposed to 500 µM ibuprophen and the expression levels of hsa miR-22, hsa- miR-4290 and hsa Let-7 were examined through quantitative real time-PCR compared to untreated MKN-45 cells. GAPDH was used as a control gene. Each column in the graph was the mean ± SEM of three independent experiments. (ns = non- significant, *P < 0.05, **p < 0.01, ***p < 0. 001) (GraphPad Prism 6 software).

cDNA, respectively. Then, the expression of microRNAs was evaluated by real-time PCR. As illustrated in Fig. 3, ibuprofen treatment increased the expression level of miR-4290, miR-22 and Let-7 by 2.14, 57.8 and 1.58 folds, respectively, relative to control MKN-45 cells. Increased level of miR-4290, miR-22 and Let-7 in MKN-45 cells exposed to ibuprofen indicated the tumor suppressor characterization of these microRNAs in gastric MKN-45 cells. In addition, cell death-induced ibuprofen seems to be primarily mediated by enhancement of the level of miR-22.

Predicted Targets of microRNA

By prediction using bioinformatic algorithms obtained from targetscan (http://www. targetscan.org) and mirbase targets (http://microrna.sanger.ac.uk/cgi-bin/targets/v5/search.pl), and miRWalk the potential binding sites and targets of hsa-miR-22 (position2659e2680), hsa-miR-4290 and hsa-Let-7 are predicted and were searched in KEGG database to identify the signaling pathway related to targets of microRNAs. We found that among targets, miR-22 and hsa-Let-7b seems to reduce the expression levels of oncogenes while hsa-miR-4290 has both oncogenic included GPCR and RAR as well as tumor suppressive targets as Axin, CDKN2, T15, Ptch, LKB1 and P15. Among them, GPCR and RAR are considered as the main regulator of WNT signaling pathway. In addition, the involvement of tumor suppressors of ARF in p53 signaling; Axin in WNT signaling; P15 in TGF-β pathway and cell cycle;Ptch in hedgehog pathway; LKB1 in mTOR; PI3K-Akt and MAPK was found through KEGG software.

DISCUSSIONS

Nonstroidal anti-inflammatory drugs (NSAIDs) including ibuprofen are able to suppress the growth of cancer cells. The molecular mechanism underlying anti-proliferative activity of NSAIDs is still unclear. The aim of this study was to evaluate the effect of ibuprofen on the expression level of three microRNAs in MKN-45 gastric cancer cells. It has been suggested that the expression of genes involved in regulation of cell cycle, cell proliferation, cells death and cell-cell communication was changed in response to exposure of cancer cells to NSAIDs [30]. First, we evaluated the growth inhibitory effect of ibuprofen on MKN-45 using trypan blue staining. Time- and dose-dependent reduction of MKN-45 gastric cancer cells treated with ibuprofen was in accordance with previous studies [19,27,31,32]. Then, results showed that the antineoplastic

effect of ibuprofen was mediated by altered expression of some miRNAs such as miR-22, miR-4290 and Let-7. In one study, the expression profile of miRNAs was explored in gastric cancer stem cells (CSCs) derived from MKN-45 cancer cells. Among them, nine microRNAs including miR-4290 was overexpressed and the others including Let-7 and miR-22 were underexpressed in spheroid body forming MKN-45 cells [17]. Mir-4290 was located in chromosome 9 in cytogenetic banding 9q22.2. There are a few reports regarding miR-4290 and its function in literature. In one study, upregulated expression of miR-4290 in K562 derived exosomes was reported as compared to K562 cells [33]. The other study has been found the elevated level of miR-4290 in MKN-45 cancer stem cells [17]. However, we found that the expression level of miR-4290 was augmented in MKN-45 cells in response to ibuprofen treatment. That is, ibuprofen–induced miR-4290 involved in the regulation of genes modulating cell death and miR-4290 acts as a tumor suppressive miRNA. To further evaluation, targets of miR-4290 was studied using three online soft wares including target scan, miR walk and miRANA which elucidated the dual role of has-miR-4290 in MKN-45 cells-derived CSCs. Oncogenic targets were included GPCR and RAR while Axin, CDKN2, T15, Ptch, LKB1 and P15 were determined as tumor suppressive targets for hsa-miR-4290. GPCR and RAR are considered as the main regulator of WNT signaling pathway. In addition, the involvement of tumor suppressors of ARF in p53 signaling; Axin in WNT signaling; P15 in TGF-β pathway and cell cycle; Ptch in hedgehog pathway; LKB1 in mTOR; PI3K-Akt and MAPK was found through KEGG software. It seems that downregulation of oncogenes such as GPCR and RAR-mediated miR-4290 overexpression plays the important role in cytotoxic effect of ibuprofen in MKN-45 cells. MiR-22 on the short arm of *chromosome* 17 has identified to reduce in gastric cancer stem cells [34,35]. In addition, CD151 has been shown to be as a target of miR-22 that is significantly inhibited in cells overexpressing miR-22 [35]. CD151 is commonly elevated in cancer cells [36-38] and is involved in the regulation of motility and metastasis both *in vitro* and *in vivo* [35,36,39]. Besides, Akt signaling factor was shown to be able to increase the expression of miR-22 [40]. In this line, microarray analysis showed that the expression of miR-22 was reduced in CSCs derived from MKN-45 cells.

Our results indicated the increased expression of miR-22 in MKN-45 cells treated with ibuprofen. Therefore, miR-22 is anticipated as a suppressor of oncogenes in response to ibuprofen treatment. Our results is in agreement with the results of [17] that miR-22 acts as tumor suppressor in gastric cancer cells. The gene locus of hsa-Let-7b was located on chromosome 22. It has been shown that c-MYC reduced the level of has-Let-7b while PIGF increased the level of has-Let-7b [41,42]. Our results showed that ibuprofen increased the level of has-Let-7 that was previously evaluated as a tumor suppressor in gastric cancer stem cells [17].

CONCLUSIONS

Taken together, our results indicated that ibuprofen is able to reduce the proliferation of MKN-45 gastric cancer cells. Anti-proliferative effect of ibuprofen may be associated to augmentation of the expression of hsa-miR-4290, hsa-miR-22 and hsa-Let-7b; this is a big claim. It is not concluded that the anti-proliferative effect of ibuprofen is mediated by hsa-miR-4290, hsa-miR-22 and hsa-Let-7b. They may be few mediators and we are blind for the rest mediators.

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