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Investigating the Effect of Ibuprofen on DLL1 and NOTCH1 Expression in Gastric Cancer Stem Cells Derived from MKN-45 Cell Line

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Abstract

Background: Cancer stem cells (CSCs) harbor the self-renewal properties of the embryonic stem cells in addition to differentiation. While maintaining the balance between self-renewal and differentiation is required for homeostasis, dysregulation in the stem cell signaling pathways such as Notch signaling increases the proliferation of these cells. As a result, tumorigenic properties are obtained. Therefore, it appears that by targeting the CSCs, the generation of new cells could be reduced and the tumor could be contained. Ibuprofen is one of the nonsteroidal anti-inflammatory drugs that are in use to control pain and inflammation. They inhibit cyclooxygenase 1, 2 (COX1,2) activity and synthesis of prostaglandins. Recent studies have provided some evidence for the anti-tumor activities of NSAIDs through inhibition of cell proliferation.

Methods: In this study, we investigate the changes in the expression patterns of two key genes, i.e., DLL1 and NOTCH1, which are involved in the Notch signaling pathway in the MKN-45 derived gastric CSCs treated with ibuprofen.

Results: Our results showed that ibuprofen up-regulates the expression of the DLL1 gene (4.1 fold) and reduces 68% the transcript level of the NOTCH1 gene (*P*-value < 0.05). These findings show that in gastric CSCs, NOTCH1 gene may act as an oncogene and, conversely, DLL1 gene may act as a tumor suppressor gene.

Conclusion: Our findings suggest that ibuprofen, by targeting CSCs, may be used as an adjuvant chemotherapy drug to improve gastric cancer treatment outcomes.

Keywords: Notch signaling pathway, Nonsteroidal anti-inflammatory drugs (NSAIDs), Gastric cancer, Cancer stem cells (CSCs)

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Introduction

Gastric cancer is the third fatal disease worldwide.[1] Numerous approaches have been developed for the treatment of gastric cancer. However, due to recurring tumor and metastasis, diagnosis and treatment of this disease are poor.[2] Therefore, new approaches are required to improve the treatment of gastric cancer.

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Recent studies have suggested that within a minority of tumors, there is a population of cells termed cancer stem cells (CSCs).[3] CSCs with their self-renewal property might be responsible for tumor initiation, progression, metastasis, recurrence, and treatment resistance.[4] Therefore, targeting these cells could be an alternative approach for cancer treatment. According to studies, gastric CSCs markers include CD26, CD44, CD133, ALDH1, and LGR5+ cells.[5] Another gastric stem cell marker is double cortin-like kinase (Dclk1), which is highly expressed in the mouse gastric CSCs model.[6] Identifying changes in gene expression that are treated with special medication can be useful for relieving tumor proliferation.[7]

A notch-signaling pathway plays an important role in the formation and evolution of different tissues. Initiating this pathway needs the cell-to-cell binding.[8] Four receptors (notches 1-4) and five membrane ligands (Jagged-1, Jagged-2, Delta-like-1, Delta-like-3, and Delta-like-4) have been identified in mammals.[9] This pathway also plays a role in determining the fate of the cells, including differentiation, progression, of the cell cycle, preservation, and self-renewal of stem cells.[10] Depending on the tissue type, several membranes of the notch signaling act either as oncogenic or tumor suppressor. Therefore, the malfunction of this pathway may contribute to the progression of several types of malignancy.[11] The most malignancy that has been studied in this regard is related to acute lymphoblastoma, leukemia and various types of cancers such as gastric, breast, brain, ovary, and lung.[12]

Nonsteroidal anti-inflammatory (NSAIDs) are the most frequent drugs mainly used for the treatment of pain, fever, and inflammation.[13,14] Furthermore, recent studies have shown that these drugs can act as antitumor and reduce the tumorigenic activity. However, the mechanisms by which NSAIDs affect the CSCs are still unknown.[15] NSAIDs inhibit COX activity; consequently, the synthesis of prostaglandin, one of the most important intermediates of inflammation, would be reduced. COX enzymes are necessary to the conversion of arachidonic acid to prostaglandin H2, a precursor in the synthesis of prostaglandins (PGs). Therefore, using NSAIDs has some side effects because prostaglandins are the main source of pain, fever, and inflammation.[13,16] Examples of some important NSAIDs are ibuprofen and aspirin. Ibuprofen is commonly used to reduce pain and inflammation.[17] In addition, ibuprofen modulates various CSCs pathways including the Notch signaling pathway by inducing apoptosis. Thus, this drug could improve the treatment for various cancers.[18] Despite several studies on the effect of ibuprofen in most cancers, to our knowledge, there is no investigation on the effect of ibuprofen in gastric CSCs. In the present study, the impacts of ibuprofen on the expression of DLL1 and Noth1 in gastric CSCs derived from MKN-45 cells are studied.

Materials and methods

Cell culture

MKN-45 cells were prepared from the Pasteur Institute of Iran. MKN-45 cells were cultured in Dulbecco's Modified Eagle's Medium/F12 (Sigma-Aldrich) containing 10% of Fetal Bovine Serum (FBS) (Gibco) and Streptomycin at 100 µg/mL and penicillin 100 unit/mL. Cells were grown at 37°C, 5% CO_2, and 95% humidity. The culture medium was changed twice a week.

Isolation of CSCs

To isolate the CSCs, 30,000 MKN-45 cells were counted at the growth phase with trypan blue and added to the cellular flasks with 25 cm diameter without adhesion. The flasks contained 5 mL of DMEM/F12 supplemented with 5% FBS. The cultures were incubated for 14 days under in the presence of 5% CO_2 and 95% humidity at 37°C The culture medium was changed twice a week. Continuous colony formation was monitored using phase-contrast microscopy.

Cell viability assay

The isolated CSCs were treated with ibuprofen for 24 h. The viability of cells was measured using Neutral Red. For this purpose, 40,000 stem cells were seeded in the plate and treated with

different concentrations of ibuprofen including 400, 600, 800, 1000, and 1200 µM. The following formula was used to convert the Optical Density (OD) measurements of each concentration to the percentage of live cells:

Percentage of bio-viability = OD Treatment/OD control×100

The concentration of ibuprofen that reduced the percentage of living cells by half was considered as the effective concentration of ibuprofen for the gene expression study.

RNA extraction and cDNA synthesis

To assess the expression levels of DLL1 and Notch1, total RNAs were extracted from cultured cells using the Trizol reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instruction. Next, the quality and quantity of RNA samples were determined by the optical density at 260/280 nm ratio. The cDNA samples were prepared for the treatment and control samples using Prim Script reagent kit (Takara, Japan).

The quantitative real-time PCR assay

First, gene-specific PCR primers were designed via online primer design software (Primer3, IDT, and Primer-BLAST) for DLL1 and NOTCH1. The sequence of primers for the DLL1, NOTCH, and GAPDH is given in table 1. Real-time polymerase chain reactions were performed using SYBR master mix and specific PCR primers. The relative changes in the gene expression levels were calculated by Livack method.[19]

Statistical Analysis

Cell viability assay and quantitative real-time PCR assay were performed at least in duplicate and their results were analyzed by Student's t-test in SPSS software version 17. Data were presented as the mean±S.E.M and the *P*-value significance was less than 0.05.

Results

Ibuprofen inhibits CSCs derived from MKN-45

The varied concentration of ibuprofen including 400, 600, 800, 1000, 1200 µM after 48 h revealed that cell viability of CSCs derived from MKN-45 decrease in a time and concentration-dependent manner (Figure 1). Viability for these concentrations was 95%, 82%, 78%, 49%, 42%,

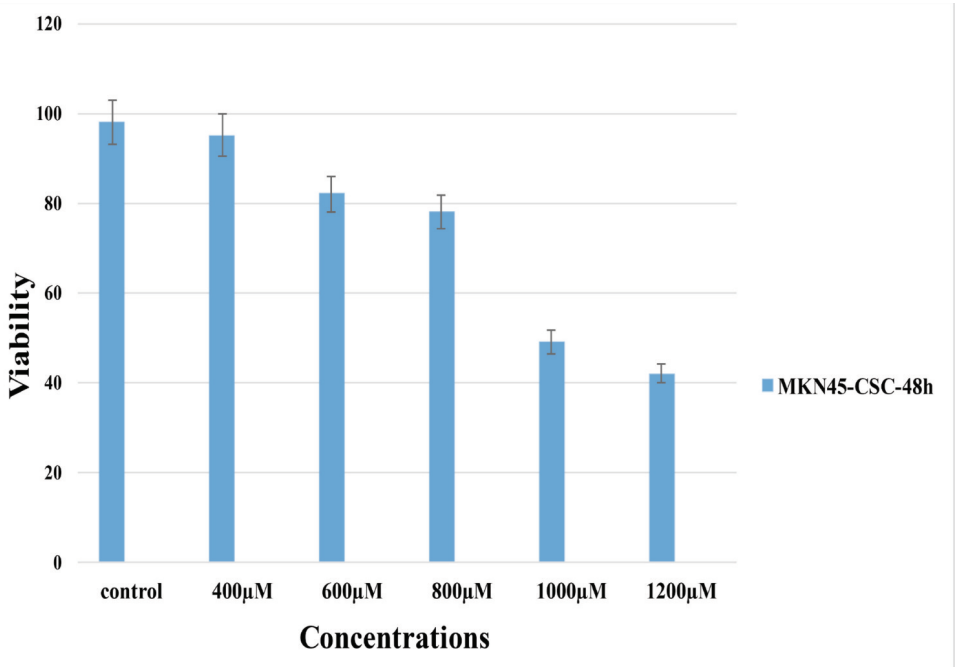

Figure 1. The effect of ibuprofen on CSCs viability; Cell viability of cancer stem cells derived from MKN-45 and treated with 400, 600, 800, 1000, and 1200 µM ibuprofen for 48 h in a six-well plate; Cell viability was evaluated via trypan blue staining. The assay was organized in three independent triplicate experiments. Error bars are shown as mean ± SEM (*P*-value < 0.05, ANOVA analysis).

Table 1. Primer sequences required for RT-Real time PCR

Genes Name	Primes	Sequences (5´->3´)	Length(bp)	Tm	GC%	Product length(bp)
NOTCH1	Sence	TGCCTGGACAAGATCAATGAG	21	58.00	47.62	143
	Antisence	CAGGTGTAAGTGTTGGGTCC	20	58.11	55.00	
DLL1	Sence	GTACTGTGACGAGTGTATCCG	21	57.95	52.38	143
	Antisence	TCTTGCAGGGCTTATGGTG	19	57.42	52.63	
GAPDH	Sence	ACTCTGGTAAAGTGGATATTGTTGC	25	54		162
	Antisence	GGAAGATGGTGATGGGATTTC	21	54		

*bp= Base pair; Tm= Primer melting temperature; GC= Guanine-Cytosine

respectively. The IC50 value of ibuprofen in CSCs derived from MKN-45 was 1000 µM at 48 h (*P*-value < 0.05). We observed that the cell viability was decreased at all concentrations used. However, using 1000 µM of ibuprofen showed a decrease in viability by 50%. This concentration was used as IC50 for cells treatment in order to measure the gene expressions. Viability of untreated CSCs with 1% DMSO was measured to be about 98%.

Ibuprofen suppresses notch pathway activity in gastric CSCs

Treatment with 800-µM ibuprofen for 48 h changed genes expression level and influenced the NOTCH signaling pathway in CSCs derived from MKN-45 CSCs. To find whether ibuprofen targets gastric CSCs via inhibition of Notch pathway activity, we investigated the expression of DLL1 and NOTCH1 by Real-Time PCR. Our analysis showed that ibuprofen treatment increased DLL1 4.1-fold in CSCs derived from MKN-45 cell line while NOTCH1 decreased it for about 68% (*P*-value < 0.05) (Figure 2).

Discussion

The results of this study show that a part of the tumor population harbors self-renewal properties of stem cells, and therefore, is labeled as CSCs. CSCs are known to be resistant to chemotherapy drugs that could be a reason for tumor recurrent.[20,21] In light of this understanding, targeting the CSCs can reduce the production of new cells in tumors.[22] Previous studies have proved that chemicals including ibuprofen, aspirin, sulindac, phosphosulindac, metformin, and celecoxib can target CSCs through inhibiting

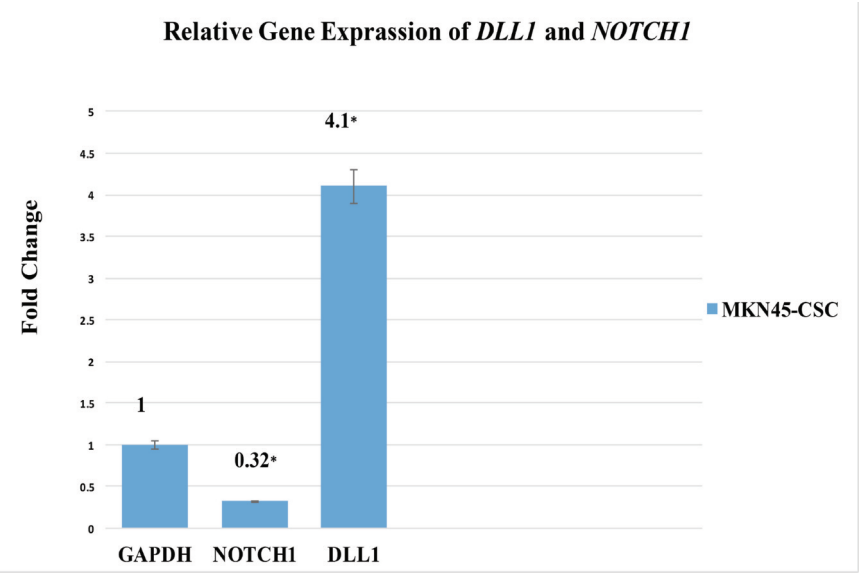

Figure 2. Effect of ibuprofen on the expression level of DLL1 and NOTCH1; RT-PCR analysis shows that the transcript of NOTCH1 decreased in CSCs affected with 800 µM ibuprofen for 48 h. In contrast, transcript of DLL1 increased. The assay was organized in three independent triplicate experiments (**P*-value <0.05, Student's test analysis).

different pathways. Ibuprofen is one of the most frequently used NSAIDs for controlling CSC signaling.[23] Several studies have indicated that prolonged use of NSAIDs has some side effects in the gastrointestinal system including bleeding and perforation, especially in females. However, a meta-analysis of data showed that ibuprofen in comparison with diclofenac, naproxen, piroxicam, and indomethacin had the lowest effect on the development of gastrointestinal injury such as gastrointestinal bleeding.[24]

Overall, the main objective of this study is to investigate the impacts of ibuprofen on proliferation and gene expression level in gastric CSCs derived from MKN-45 cell line. Our results were in agreement with other studies on the inhibitory effect of ibuprofen that had decreased proliferation and gene expression in gastric CSCs.[25,26,31] Also, it has been shown that viability in CSCs derived from MKN-45 cell line decreased with ibuprofen in time and concentration dependent-manner in contrast to untreated CSCs.[26] We investigated the effect of ibuprofen on genes expression level that plays a role in the Notch signaling pathway. In the present study, we demonstrated that ibuprofen targets gastric CSCs by up-regulating DLL1 and down-regulating NOTCH1, consequently suppressing Notch pathway activities. It has been shown that DLL1 decreases and NOTCH1 increases in gastric cancer.[27] DLL1 is a tumor-suppressor gene that acts as a ligand in the Notch signaling pathway. DLL1 plays a role in cell fate decision and prevents cancer. Lack of DLL1 in cancer cell leads to uncontrollable cell proliferation. According to KEGG pathway, DLL1 can activate hairy and enhance split1 (Hes1) genes, a downstream target of Notch signaling.[28] So, upregulation of DLL1 by ibuprofen is considered to be useful in the treatment of CSCs.

Furthermore, NOTCH1 is an oncogene that acts as a receptor in the NOTCH signaling pathway. In normal cells, the expression level of NOTCH1 is controlled, which leads to expression of Hes and Hey indirectly, Notch target genes, and activation of angiogenesis. In contrast, in cancer cells, NOTCH1 expression is increased and activated without ligand. Ibuprofen decreased NOTCH1 expression in CSCs. Thus, mRNA levels of Hes and Hey decreased and prevented angiogenesis. Accordingly, ibuprofen can be considered as a good option for treatment of CSCs and targeting these genes can be useful for inhibition of gastric cancer.[29] According to another study, ibuprofen reduces cell proliferation in gastric CSCs through inhibiting Wnt/B catenin signaling pathway.[25] As a result of this experiment, SOX2 and KLF4 were upregulated. In contrast, OCT3/4, Nanog, and CD44 were downregulated in both AGS and MKN-45 CSCs. On the other hand, WNT1, CTNNB1, and PYGO2 decreased, while KREMEN1, CTNNBIP1, and SUFU increased in both cell lines. Interestingly, SMARCD1 and CASK decreased in AGS but increased in MKN-45.[30] Also, another previous study by Hung et al. showed that celecoxib as an NSAID can reduce the proliferation of MDA-MB-231 and MCF-7 cells in breast CSC line.[31] There is a study that reported aspirin inhibits NF-KB activity and breast CSC properties.[32] In the same study, it was found that aspirin activates apoptosis pathway and increases cytoplasmic NFKB in BxPc-3, PANC-1, BxPc-3/GEM, and APC-1 cell lines derived from pancreatic ductal adenocarcinoma (PDA).[33] Likewise, it has been reported that Phosphosulindac (OXT-328) selectively targets CSCs *in vitro*.[34]

In light of the evidence obtained from different studies, it can be stated that CSCs can be a target for cancer treatment. Our result provides evidence that ibuprofen can be used to inhibit proliferation of gastric CSCs derived from MKN-45 cell line.

Conclusion

In summary, we showed for the first time that ibuprofen targets gastric CSCs by upregulation of DLL1 expression and down-regulation of Notch1 expression. Our results demonstrate that ibuprofen has CSCs inhibitory effects in gastric CSCs through increasing expression of DLL1 gene and decreasing expression of NOTCH1 gene. Therefore, ibuprofen may be used as an adjunctive

treatment to improve the efficacy of conventional chemotherapy in gastric CSCs.

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Conflict of Interest

None declared.

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