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Up regulation of *KAI1* gene expression and apoptosis effect of imatinib mesylate in gastric adenocarcinoma (AGS) cell line

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ABSTRACT

Objective: To evaluate the effect of imatinib mesylate on *KAI1* gene expression and apoptosis properties in human gastric carcinoma AGS cell line.
Methods: Cell viability was assessed by MTT assay and quantitative real time PCR method was applied for investigation of *Bax*, *Bcl-2*, and *KAI1* gene expression in AGS cells. The quantity of *KAI1*, *Bax*, and *Bcl-2* compared to *GAPDH* gene expressions were examined using the formula $2^{-\Delta\Delta Ct}$. Furthermore, cell apoptosis/necrosis was carried out by annexin V/PI staining and quantified with flow cytometry after treatment with imatinib.
Results: Imatinib mesylate was showed to have a dose-dependent toxicity effect against AGS cells. *KAI1*/*GAPDH* gene expression ratios were 1.07 ± 0.02 ($P > 0.05$), 1.68 ± 0.19 ($P > 0.05$), 3.60 ± 0.55 ($P < 0.05$), 6.54 ± 0.27 ($P < 0.001$) for 20, 50, 80 and 100 μmol/L of imatinib concentrations. The mRNA levels of *Bax* detected by real-time PCR after treatment with imatinib mesylate were significantly increased. Also, the number of apoptotic cells was increased from 3.72% (statistically significant; $P < 0.05$) in untreated AGS cells to 21.72%, 83.04% and 85.80%, respectively, following treatment with 20, 40, and 60 μmol/L imatinib mesylate.
Conclusions: The results suggest that imatinib mesylate can induce apoptosis pathway in a dose-dependent mode and might modulate metastasis by up regulating *KAI1* gene expression in human gastric carcinoma AGS cell line.

1. Introduction

Gastric cancer is the second leading cause of cancer related death worldwide[1,2]. Despite surgery remains the usual procedures for treatment of gastric cancer, its survival rate is less than 33%. The failure of conventional radio- and chemo-therapy and increasing incidence and mortality caused by advanced invasive gastric cancer requires an urgent need to explore novel agents and new targets in the search for more productive treatment[3]. Nowadays, different preclinical and clinical studies on molecular targeted therapies have exhibited that it has great promise in the treatment of several malignant tumors[4]. Imatinib mesylate (Gleevec, Novartis, Basel, Switzerland) was first used as a new class of agents that inhibit specific tyrosine kinases. Imatinib mesylate has been shown to compete with adenosine triphosphate and inhibit specific tyrosine kineses such as Bcr-Abl kinase, c-kit receptor kinases activation as well as inhibit platelet-derived growth factor (PDGF) (α, β) receptor[5-8]. Moreover, numerous studies have been conducted to date demonstrating the inhibitory effect of imatinib on the growth of variety of tumor cell types, such as of lung, pancreatic, osteosarcoma, myeloid, thyroid, and ovarian origin and is presently utilized in research and treatment of several malignant tumors[9]. In

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cancer cells, cell cycle control become deregulated and cells have an imbalanced proliferation to apoptosis ratio. Resistance to apoptosis is one of the hallmarks of human cancers, thus induction of apoptosis in malignant tumor cells is a common method for cancer therapy[10].

Bone metastasis is a common problem of cancer patients, with up to 70% of patients due to malignant cancer[11]. Thus, substantial attention has been focused on the gene expression studies in cancer cells and it has been the topic of intense method for inhibition of tumor progression and targeting metastatic cascade. *KAI1/CD82*, a tumor metastasis suppressor gene, is known a significant play in targeting metastasis of tumor. Down-regulation of the *KAI1* family genes has been associated with a variety of cells with a high potential for metastasis[12,13]. Many investigations have confirmed that the *KAI1/CD82* gene suppresses metastasis in different types of cancers involving cervical, endometrial, pancreatic, ovarian, lung, hepatic, gastric, colorectal and esophageal and breast cancer[14,15]. To our knowledge, the aim of present work was to evaluate anti-cancer effect through modulation of *KAI1/CD82* gene expression in gastric carcinoma cell line. Also, the assessment of apoptotic/necrosis activity of imatinib mesylate in gastric AGS cancer cell is also investigated.

2. Materials and methods

2.1. Cell culture and Imatinib treatment

Human gastric adenocarcinoma (AGS) cells were purchased from Pasteur Institute of Iran. Cells were maintained in RPMI-1640 supplemented with fetal bovine serum, 100 μg/mL streptomycin and 100 IU/mL penicillin (all purchased from Sigma, USA). The AGS cell line was distributed into 96-well plates with 1×10^4 cells/well. Various concentrations of imatinib (0, 10, 20, 30, 40, 80 and 100 μmol/L) were treated into grown cell for overnight at 37 ˚C. In each concentration, imatinib mesylate was incubated on six wells of 96-well plate cells.

2.2. Cell viability by using MTT assay

The cell viability was determined by MTT assay. Briefly, 10 μL of the MTT dye solution (5 mg/mL) was added to each well, followed by treatment for 4 h at 37 ˚C. The insoluble purple formazan crystals formed within living cells were solubilized by adding isopropanol (Merck, Germany). Finally, the optical density (OD) of wells was measured at 570 nm by using a plate reader (Organon Teknika, Netherlands). The percentage of cell viability related to control (untreated) was calculated according to following equation:

$$\text{Viability (\%)} = \frac{\text{OD value of test samples}}{\text{OD value of test controls}} \times 100$$

2.3. RNA isolation and cDNA synthesis

The AGS cell line was seeded into six well plates (5×10^4 cells/well) and incubated for 24 h, and then, the cells were treated with imatinib for another 24 h. Total RNA was extracted using the RNA-isolation kit (Qiagen, RNeasy Plus Mini Kit 50) based on manufacturer's protocol. RNA isolated with 260/280 nm absorption ratio > 1.8 was subjected for cDNA synthesis. cDNA synthesis was amplified utilizing Prime Script™ 1st strand cDNA synthesis kit (Takara, Japan) at total volume of 20 μL, containing 4 μL Prime Script™ buffer 5×, 3 μL of total RNA (1 μg), 1 μL oligo dT primer (50 μmol/L), 1 μL of dNTP mix (10 mmol/L), 1 μL random 6 mers (50 μmol/L), 1 μL Prime Script™ RTase (200 units), 1 μL RNase inhibitor (40 units) and 8 μL nuclease-free water. Thermal cycling was incubated within 10 min at 30 ˚C, for 50 min at 42 ˚C and 5 min at 95 ˚C and afterwards cooled to 4 ˚C within 5 min.

2.4. Real-time-PCR

A SYBR Green real-time PCR was carried out to quantify the amount of mRNA level from AGS cells. The expression of target genes were quantified by using an ABI 7300 real-time PCR system under following conditions: 95 ˚C for 10 min, 40 cycles were followed by 95 ˚C for 15 s and 60 ˚C within 1 min. Each PCR amplification reaction was carried out in 20 μL reaction mixture containing 2 μL cDNA sample (100 ng), 1 μL of primers (0.4 mmol/L), 10 μL Power SYBR Green PCR Master Mix (2×), and 6 μL double-distilled water. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) used as an internal standard control gene for real time PCR. The gene expression was calculated by comparative threshold cycle (Ct) values throughout the exponential phase of amplification. Thereafter, mCt (mean threshold cycle) values of *GAPDH* was subtracted from mCt value of *KAI1*, *Bax* and *Bcl-2* genes to achieve ΔCt and ΔΔCt of samples were computed from Ct corresponding values; where ΔΔCt = [mCt *KAI1* – mCt *GAPDH*] $_{\text{(normal sample)}}$ – [mCt*KAI1* – mCt *GAPDH*]$_{\text{(test sample)}}$. Eventually, Targets/reference gene expression ratio was measured employing the ratio formula (gene dosage ratio = $2^{-\Delta\Delta Ct}$). The sequence of real-time PCR for desire primers were as following:

KAI1: sense: 5′-CTCAGCCTGTATCAAAGTCACCA-3′
Anti-sense: 5′-CCCACGCCGATGAAGACATA-3′
Bax: sense: 5′-TTGCTTCAGGGTTTCATCCAG-3′
Anti-sense: 5′-AGCTTCTTGGTGGACGCATC-3′
Bcl-2: sense: 5′-TGTGGATGACTGAGTACCTGAACC-3′
Anti-sense: 5′-CAGCCAGGAGAAATCAAACAGAG-3′
GAPDH: sense: 5′-CGTCTGCCCTATCAACTTTCG-3′
Anti-sense: 5′-CGTTTCTCAGGCTCCCTCT-3′

For primer specificity, it has been tested by using BLAST program (www.ncbi.nlm.nih.gov/blast). The specificity of all amplification reactions were approved by melting curve analysis.

2.5. Flow cytometric analysis of apoptosis/necrosis

Apoptotic cells were stained by using Annexin-V- FLUOS and propidium iodide (PI) staining kit (Roch, Germany) according to the manufacture's instruction. AGS cells (1×10^5 cells/well) were treated with imatinib mesylate at the desired dose for overnight. Finally, the numbers of apoptotic/necrotic cells were calculated by flow cytometry using PAS machine (Partec, Germany). Each experiment was carried out in triplicate.

2.6. Data analysis

The results were exhibited as mean ± SEM of values obtained deviation and the experimental data were examined by using SPSS version 22. $P < 0.05$ was considered as a significant.

3. Results

3.1. MTT assay results

Various concentrations of imatinib mesylate had cytotoxicity effect on cancer cell line at 24 h. At concentration of 10, 20, 30, 40, 80 and 100 μmol/L of imatinib, the viability of AGS was reduced to (84.24 ± 0.02)%, (68.08 ± 0.07)% (statistically insignificant, $P > 0.05$), (45.25 ± 0.08)% ($P < 0.05$), (29.89 ± 0.01)% ($P < 0.05$), (21.21 ± 1.01)% ($P < 0.05$) and (8.4 ± 1.05)% ($P < 0.01$), respectively (Figure 1). The IC_{50} value of imatinib on gastric cancer AGS cell line was calculated 41.19 μmol/L.

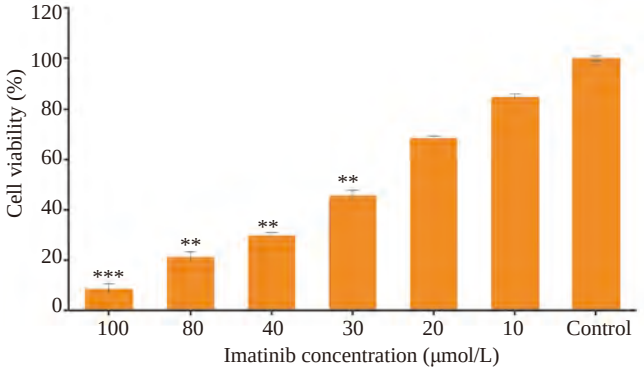

Figure 1. Cell viability assay of AGS cells 24 h after treatment with Imatinib mesylate.
All data were described as mean ± SEM from three independent experiments. Percentages of viability were expressed relative to untreated control. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

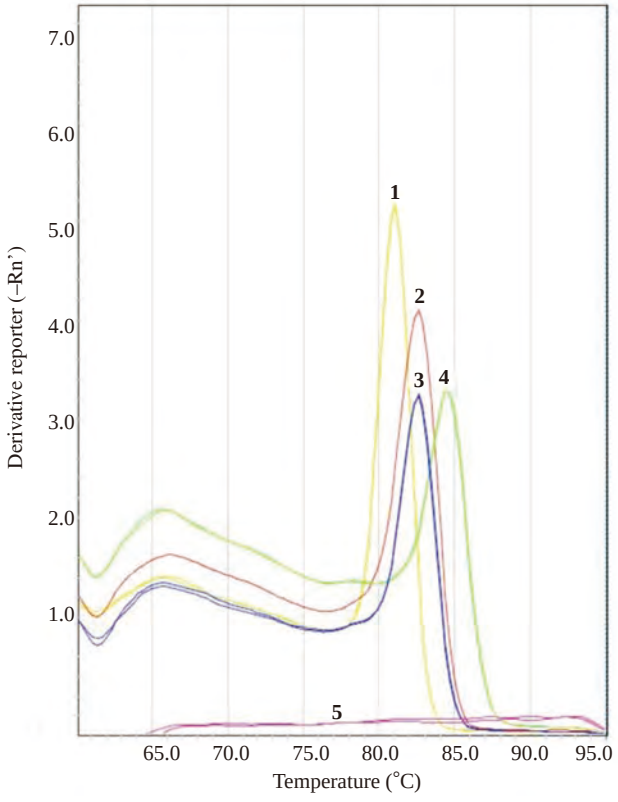

Figure 2. Melting curve analysis for PCR products obtained with the specific primer pairs for *Bax*, *Bcl-2*, *KAI1*, and *GAPDH* genes.
The melt curve peaks at 81.04 °C assign to *KAI* (1), 83.2 °C for *GAPDH* gene (2), 83.5 °C for *Bcl-2* (3), 84.08 °C for *Bax* gene (4) demonstrate that the specific products melted at various temperatures. Flat peak indicates non-template control (5).

3.2. Melting curve analysis

The melting curve analysis was carried out at temperature (x axis) and ΔRn derivation (y axis). It was created to screen for primer dimmers of desired genes and to confirm the specificity of the amplified product. The melting peaks have been drawn for *Bax*, *Bcl-2*, *KAI1*, and *GAPDH* genes as shown in Figure 2.

3.3. Relative quantification analysis for *KAI1/CD82* gene

Evaluation of relative quantification analysis for *KAI1/CD82* gene expression between untreated and treated could be calculated by the difference in their Ct values during exponential phase of amplification. The value of mCt *GAPDH* gene was 17.32 at various concentration of imatinib mesylate (0, 20, 50, 80 and 100 μmol/L). The mCts values of *KAI1* were 29.74, 28.38, 26.80, 25.94 and 24.38 at 0, 20, 50, 80 and 100 μmol/L concentration of imatinib mesylate, respectively. The value of mΔCt for untreated sample was measured 11.16. The value of mΔCt for treated samples in different concentration of imatinib at 20, 50, 80 and 100 μmol/L were calculated 11.06, 10.40, 9.31 and 8.43, respectively. The ΔΔCt values were scaled as –0.098, –0.755, –1.840 and –2.240 for 20, 50, 80 and 100 μmol/L imatinib concentrations. The measured gene dosage ratios ($2^{-\Delta\Delta Ct}$) for *KAI1/CD82* gene were 1.07 ± 0.02 ($P > 0.05$), 1.68 ± 0.19 ($P > 0.05$), 3.60 ± 0.55 ($P < 0.05$), and 6.54 ± 0.27 ($P < 0.001$) for 20, 50, 80 and 100 μmol/L of imatinib concentrations (Figure 3).

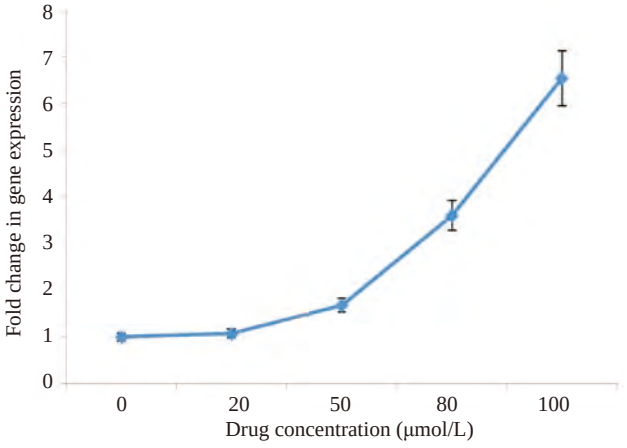

Figure 3. Up regulation of *KAI1* gene expression in human gastric adenocarcinoma (AGS) cell line treated with imatinib mesylate.

3.4. Analysis of apoptosis-related gene expression

The relative transcript abundance of the *Bax* (pro-apoptotic gene) and the *Bcl-2* (anti-apoptotic gene) in absence or present of imatinib mesylate were measured by quantitative real time PCR (Figure 4). Our finding revealed that mRNA level of *Bax* was significantly enhanced ($P < 0.05$) in the AGS cells with 40 and 60 μmol/L imatinib treatments compared to the controls. The expression of *Bax* transcripts in cells cultured with 20 mmol/L imatinib did not differ from control cells. Moreover, the expression level of *Bcl-2 gene* in 40 μmol/L imatinib treatment was significantly reduced compared to control.

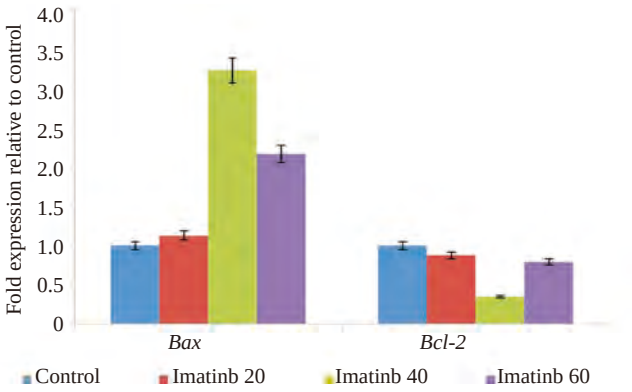

Figure 4. Relative expression levels of *Bcl-2* and *Bax* genes in gastric AGS cancer cell with or without imatinib mesylate.

Each sample was normalized to internal control (*GAPDH*) mRNA level. Up regulation of *Bax* was significantly enhanced in the cells with 40 and 60 μmol/L imatinib treatments compared to the controls. While, the expression level of *Bcl-2* gene in 40 μmol/L imatinib treatment was significantly reduced.

3.5. In vitro apoptosis/necrosis assay

To further determine imatinib-induced apoptosis of AGS cells, cells were stained with Annexin-V/PI assay, followed by flow cytometry analysis after 24 h. Within early apoptosis stage, phosphatidyl serins (PS) in cell membrane translocated from inner leaflet of cell membrane to the outer face of cell membrane. PS can be stained with an annexin-V, while PI can detect cell nucleus, which have damaged their integrity during necrosis. The number of apoptotic cells was increased from 3.72% (statistically significant, $P < 0.05$) in untreated AGS cells to 21.72%, 83.04% and 85.80%, respectively, following treatment with 20, 40, and 60 μmol/L imatinib mesylate (Figure 5).

4. Discussion

Tyrosine kinases inhibitors (TKIs) are promising anticancer agents that slow progression growth of experimental bone metastases and often induce apoptosis in neoplastic cells. Imatinib mesylate is one

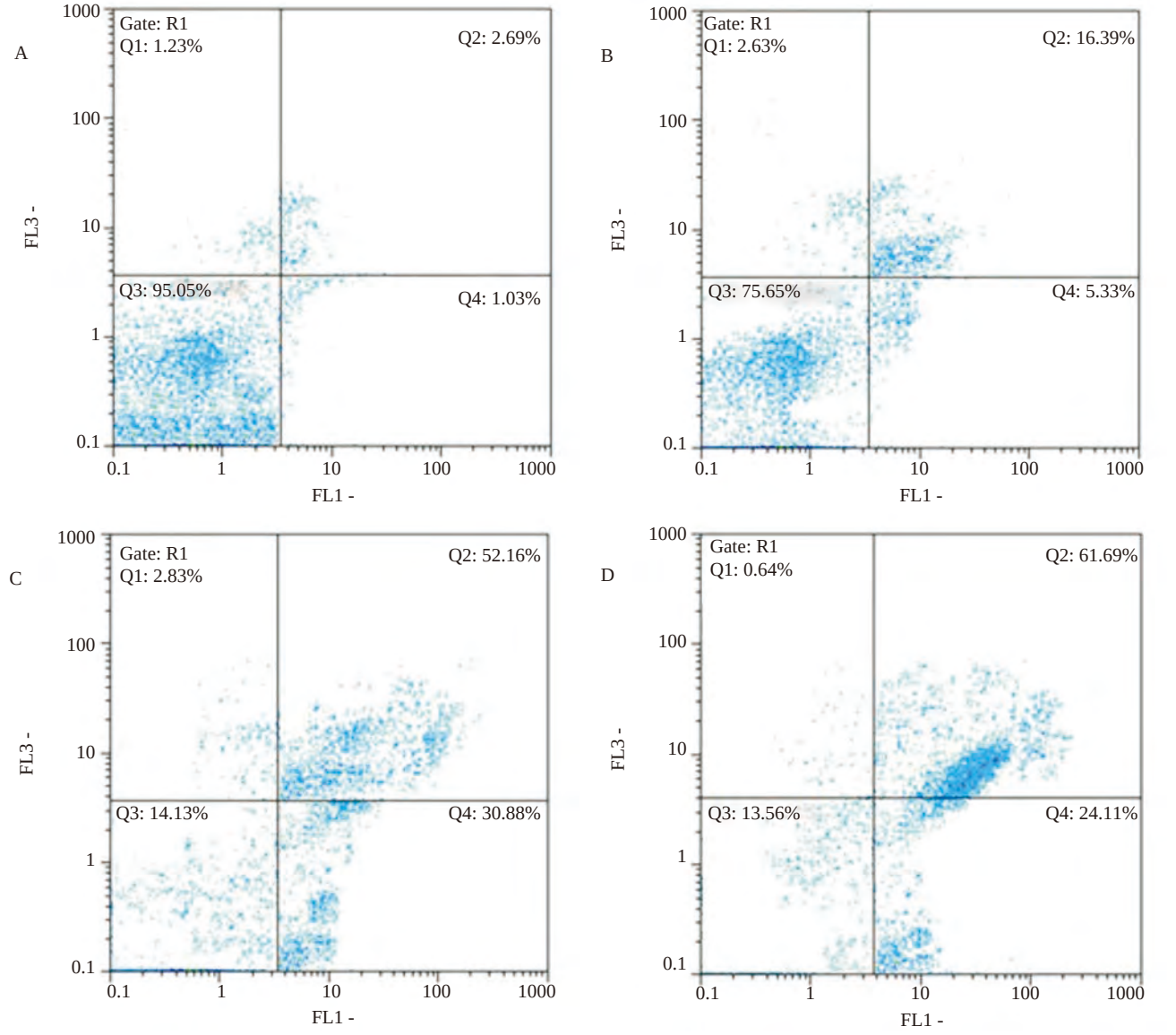

Figure 5. Flow cytometric analysis by annexin V-FLUOS (FL1) in x-axis and PI (FL3-H) in y-axis double staining of AGS cells treated with imatinib mesylate at 24 h.

Dot plots of annexin V/PI staining are shown in untreated AGS cells (A). B: AGS cells treated with 20 μmol/L imatinib exhibited 5.33% early stage apoptosis and 16.39% late stage apoptosis. C: AGS cells treated with 40 μmol/L imatinib had 30.88% early stage apoptosis and 52.16% late stage apoptosis. D: AGS cells treated with 60 μmol/L imatinib showed 24.11% early stage apoptosis and 61.69% late stage apoptosis.

of the first successful members of TKIs with activity against Bcr-Abl fusion oncoprotein, ABL, PDGFRA, and c-KIT in gastrointestinal stromal tumors and is currently under evaluation in clinical trials for ovarian cancer, prostate cancer, malignant gliomas, and carcinoid tumor[16,17].

Imatinib mesylate interferes with a different specific molecular target include cell-cycle proteins, growth factor receptors, molecules involved in invasion and angiogenesis, and modulators of apoptosis, which are necessary for development in normal tissues. In other investigations, the cytotoxic effects of imatinib on various cancer cell lines have been reported[18]. In the current work, we demonstrated that imatinib exerted a dose-dependent inhibitory manner on viability of gastric adenocarcinoma AGS cell line.

Most cancer related mortalities are caused by the progression of tumor metastasis, therefore the most significant contributor to cancer related mortality and morbidity will be due to prevention of such disseminated disease[19]. Nowadays, identification of the gene expression within progression of tumor has been the great intense procedure for therapy and prognosis[20]. *KAI1* was initially introduced as a tumor metastasis suppressor gene in prostate cancer. Later, *KAI1* was demonstrated to be a tumor metastasis suppressor gene during tumor progression in various solid tumors. It has been revealed that *KAI1* plays a significant role in down regulation of metastasis and tumor growth. In various studies of human malignant tumors like ovarian, breast, bladder, prostate, cervical and hepatocellular carcinoma, an association between decreased expression of *KAI1* and increasing metastatic ability was demonstrated[21,22]. Hinoda *et al*. has reported the reduced expression of the *KAI1* gene in MKN74 and NUGC3 gastric cell lines. They suggested that *KAI1* expression might be contrarily associated to the disease progression of human gastric cancers[23]. *KAI1* protein has reported to interact with several epidermal growth factor receptors, chemokines and intergins[24]. These associations likely play a significant function in cell-cell interaction, cell motility, and cell signaling which imply an important role of *KAI1* gene. Beside, down regulation of *KAI1* gene expression, both at the transcriptional and translational level, eventually leads to poor prognosis in clinical cases[25]. In here, we demonstrated up regulation of *KAI1* gene expression in human gastric cancer cell line treated with imatinib mesylate that has not yet been studied. These observations indicate that imatinib can probably inhibit metastasis by increasing the expression of *KAI1* gene in AGS cells. Therefore, imatinib remains a promising candidate for the treatment of gastric cancer in the future. In the current study, the expression of *Bcl-2* and *Bax* are regulated by imatinib mesylate. The expression of *Bax* increased significantly 3.23 and 2.17 fold at 40 μmol/L and 60 μmol/L imatinib mesylate concentrations, respectively. Decreased expression of *Bcl-2* at 20 and 60 μmol/L imatinib was not significant respect to control group; however *Bcl-2* showed significant expression (0.25 fold) at 40 μmol/L concentration of drug respect to control. Apoptosis is managed by anti-apoptotic and pro-apoptotic effectors that involve a broad variety of proteins. The product of the Bcl-2 family gene and other anti-apoptotic members (BCL-w, Mcl-1, A1, Bcl-2, and, Bcl-xl) serves as significant regulators of apoptosis pathways[26]. Bcl-2 protein inhibits the release of cytochrome c from mitochondria elicited by Bax proteins, resulting in repression of apoptosis cascade[27].

Previous studies have demonstrated that imatinib mesylate has cytotoxic effects and apoptosis-inducing properties in various human solid tumors cell types such as glioblastoma[28], ovarian, thyroid[29], breast cancer, dermatofibrosarcoma protruberans and documented that *Bax*, *Bad*, *Bcl*-2, and *Bcl-xl* gene expression were differentially regulated by imatinib mesylate[30,31]. Similar investigations were revealed that imatinib mesylate can be down regulated expression of anti-apoptotic proteins, involving Bcl-xl, Mcl-1, and Bcl-2 in human K562, c-KIT-dependent gastrointestinal stromal tumor and cholangiocarcinoma cancer cell lines[32]. A few studies have been investigated for anti cancer effect of imatinib mesylate on gastric cancer. Kim *et al*.[33] showed that imatinib mesylate can promote apoptosis of gastric cancer cells by both activation of caspase-9 and -3 and including poly-ADP-ribose polymerase cleavage. They also reported that inhibition of platelet-derived growth factor receptor expression by semi-quantitative PCR leads to promoting apoptosis in gastric cell lines[33]. To evaluate if imatinib mesylate treatment results in apoptosis induction, our results revealed that imatinib induces an intrinsic signaling pathway for programmed cell death mediated by down regulation of *Bcl-2* and up-regulation of *Bax* gene expression on gastric cancer AGS cell line, which have not been reported so far.

Further investigation made in this work was Annexin-V with PI staining, followed by flow cytometry. During the early apoptosis, PS in membrane translocated from inner leaflet into the cell surface. Annexin-V/PI assay could be used for detecting the exposed PS by flow cytometry. Annexin-V stains PS of the early and late apoptotic cells, while propidium iodide (PI) detect the nucleus of the cells, which have disrupt their integrity in the necrosis. Therefore, the annexin V^+/PI^- cells detect early stage of apoptosis and annexin V^+/PI^+ cells exhibited late stage of apoptosis[34,35]. Based on annexin V/PI staining results, many annexin V^+/PI^+ cells were detected in the cells treated with 40 μmol/L and 60 μmol/L of imatinib, showing the late stage of apoptosis. While in the cells treated with 20 μmol/L of imatinib in addition to annexin V^+/PI^- cells, some annexin V^+/PI^+ cells were also monitored, showing some cells in the early stage of apoptosis. In addition, flow cytometric analysis observed that the number of early and late apoptotic AGS cells was significantly increased in a concentration dependent manner, following treatment with 40 μmol/L and 60 μmol/L of imatinib as compared to untreated AGS cell line. Our results revealed that cytotoxicity of imatinib toward AGS cells was chiefly brought about by its ability to induce apoptosis. In this study, we have shown that AGS cells have a dose-dependent apoptosis when they treated with imatinib mesylate, indicating that the cytotoxic effect of imatinib on AGS cells was induced by apoptosis rather than necrosis.

In conclusion, we demonstrated that imatinib mesylate induces intrinsic signaling pathway for programmed cell death mediated by down regulation of *Bcl-2* and up-regulation of *Bax* and *KAI1* gene expression in gastric AGS cancer cell line. Herein, this is the first report depicting significant apoptotic effect of imatinib mesylate against AGS cell line. Thus, it may be a good candidate for utilizing as an inhibitor of the progression of cancer cells *in-vivo* and the treatment of gastric cancer and explain the mechanisms by which imatinib mesylate acts on apoptotic signaling pathway. Based on these results, further investigation on other cell lines as well as animal tumor models and subsequent clinical studies are required.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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