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Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2015.10.006

Evaluation of imatinib mesylate (Gleevec) on *KAI1/CD82* gene expression in breast cancer MCF-7 cells using quantitative real-time PCR

Seyed Ataollah Sadat Shandiz[1*], Marjan Khosravani[2], Sepideh Mohammadi[3], Hassan Noorbazargan[4], Amir Mirzaie[1], Davoud Nouri Inanlou[5], Mojgan Dalirsaber Jalali[6], Hamidreza Jouzaghkar[1], Fahimeh Baghbani-Arani[7], Behta Keshavarz-Pakseresht[7]

[1]*Young Researchers and Elite Club, East Tehran Branch, Islamic Azad University, Tehran, Iran*

[2]*Department of Microbiology, Shiraz Azad University, Shiraz, Iran*

[3]*Department of Microbiology, Islamic Azad University, Pharmaceutical Sciences Branch, Tehran, Iran*

[4]*Department of Biotechnology, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[5]*R&D Department, Research and Production Complex, Pasteur Institute of Iran, Tehran, Iran*

[6]*Department of Microbiology, Faculty of Basic Science, Young Researcher Club, Lahijan Branch, Islamic Azad University, Lahijan, Iran*

[7]*Department of Genetics & Biotechnology, School of Biological Science, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran*

ARTICLE INFO

Article history:
Received 3 Aug 2015
Received in revised form 28 Aug, 2nd revised form 17 Sep 2015
Accepted 22 Oct 2015
Available online 14 Dec 2015

Keywords:
Imatinib mesylate
KAI1/CD82
Metastasis
Breast cancer
Real-time PCR

ABSTRACT

Objective: To evaluate the effect of imatinib mesylate on cell viability, anti cancer effect through modulation of *KAI1/CD82* gene expression in breast cancer MCF-7 cell line.
Methods: The effects of imatinib mesylate on cell viability in MCF-7 cell line were assessed using MTT assay and IC_{50} value was determined. *GAPDH* and *KAI1/CD82* were selected as reference and target genes, respectively. Quantitative real time PCR technique was applied for investigation of *KAI1/CD82* gene expression in human breast cancer MCF-7 cells. Subsequently, the quantity of *KAI1* compared to *GAPDH* gene expressions were analyzed using the formula; $2^{-\Delta\Delta Ct}$.
Results: Imatinib was showed to have a dose-dependent inhibitory effect on the viability of MCF-7 cells. *CD82/GAPDH* gene expression ratios were 1.322 ± 0.030 ($P > 0.05$), 2.052 ± 0.200 ($P < 0.05$), 2.151 ± 0.270 ($P < 0.05$) for 10, 20 and 40 μmol/L of imatinib concentrations.
Conclusions: Based on the present data, imatinib mesylate might modulate metastasis by up-regulating *KAI1/CD82* gene expression in human breast MCF-7 cancer cell line.

1. Introduction

Breast cancer is the second leading cause of cancer related death worldwide among women [1,2]. The high mortality and morbidity associated with breast cancer derived from its metastasis to liver, bone and lungs. The failure of conventional chemo- and radio-therapy and increasing rate of death caused by invasive breast cancer requires an urgent need to identify novel anti cancer drug and new targets for more profitable treatments [3]. Currently, different preclinical and clinical studies on molecular targeted therapies agents have shown that it has great promise in the treatment of different malignant tumors like breast cancer [4]. Imatinib mesylate (IM) (Gleevec; Novartis, Basel, Switzerland), was first used as a novel class of agents that suppress particular tyrosine kinases. IM has been registered in adults for monotherapy of chronic myeloid leukemia by inhibiting specific

*Corresponding author: Seyed Ataollah Sadat Shandiz, Ph.D., Department of Biology, East Tehran Branch, Islamic Azad University, Tehran, Iran.
Tel/Fax: +98 21 33594337
E-mail: Atashandiz@yahoo.com

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

Foundation Project: Supported by East Tehran Branch, Islamic Azad University (Grant No. 923064).

tyrosine kinases like Bcr-Abl kinase, as well as inhibiting the induction of c-kit receptor kinases and platelet-derived growth factor (αβ) receptor which regulate majority of cellular events [5–7]. Moreover, IM is an important targeted therapy agent used widely for inhibition of tumor growth in several malignancies including ovarian, pancreatic, osteosarcoma, myeloid, thyroid and lung cancer and is recently utilized in research and treatment of other solid tumors [8,9]. Metastasis to bone is a common complication of cancer patients, with up to 70% of patients due to malignant cancer [10]. Understanding the gene expression during progression of tumor has been the topic of intense method for inhibition of tumor growth and targeting metastatic cascade. Currently, metastasis suppressor genes are found to play an important role in regulation of cell invasion and metastasis signaling. *KAI1* (*CD82*), a tumor metastasis suppressor gene, has wide-spectrum roles in targeting tumor metastasis [11,12]. Many studies have confirmed that the *KAI1/CD82* gene inhibits metastasis in various types of cancers such as endometrial, pancreatic, bladder, breast, ovarian, cervical, lung, hepatic, colorectal and gastric cancer [13–15]. The main purpose of this study is to determine the effect of various imatinib concentrations on cell viability and metastasis through modulation of *KAI1/CD82* gene expression in breast cancer MCF-7 cell line.

2. Materials and methods

2.1. Cell culture

The human breast adenocarcinoma cell line (MCF-7) was purchased from the National Cell Bank of Iran, Pasteur Institute, Tehran, Iran. Cells were maintained in RPMI 1640 medium that was supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 2 mmol/L glutamine, 1% penicillin/streptomycin (all purchase from Gibco, Scotland) in an incubator environment of 5% carbon dioxide (CO_2) atmosphere at 37 °C.

2.2. Cell viability assay

To determine cell viability, we used the MTT assay [12]. Different concentrations of imatinib ranging from 0, 1.5, 2.5, 5.0, 10.0, 20.0, and 40.0 μmol/L were treated into MCF-7 grown cells (1×10^4 cells/well) in 96-well microtiter plate and incubated for 24 h at 37 °C in order to further application. The cells were treated with 10 mL of the MTT solution (5 mg/mL in phosphate buffer) and incubated at 37 °C for 4 h. Then, for solubilization of the MTT formazan products, 100 μL of dimethylsulfoxide (Merck, Germany) were added to the wells. The optical density (OD) of each well was determined at a wavelength of 570 nm with ELISA reader (Organon Teknika, Netherlands). Finally, The OD value was determined as percentage of viability by the following formula:

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

The IC_{50} value of IM was calculated as the dose at with 50% cell death occurred relative to the untreated cells.

2.3. RNA isolation and cDNA synthesis

MCF-7 cell line was seeded in 96 well plate with 5×10^4 cells per well and incubated for overnight, and then, the cells were treated with IM. Total RNA was extracted using the RNA-isolation kit (Qiagen, RNease Plus Mini Kit 50) according to the manufacturer's instructions. The purity of the isolated RNA has been performed by using a NanoDrop machine (IMPLEN, Germany). RNA isolated with 260/280 nm absorption ratio >1.8 was subjected for cDNA synthesis. Complementary DNA synthesis was amplified using Prime Script™ 1st strand cDNA synthesis kit (Takara, Japan) in 20 μL reaction mixture containing 4 μL Prime Script™ Buffer 5×, 3 μL of total RNA (1 μg), 10 mmol/L dNTPs (1 μL), 50 μmol/L oligo dT primer (1 μL), 50 μmol/L Random 6 mers (1 μL), 1 μL Prime Script™ RTase (200 units), 1 μL RNase inhibitor (40 units) and 8 μL nuclease-free water. Relative quantitation of gene expression was measured by ABI 2720 thermal cycler (Applied Biosystems, USA) for 10 min at 30 °C, 50 min at 42 °C and 5 min at 95 °C followed by cooling on ice for 5 min.

2.4. Primer design

In the present investigation, the primers for real-time PCR of *GAPDH* and *KAI1* genes were designed using Primer Express software v3.0.1. The forward and reverse primers used for *KAI1/CD82* gene were the (5′ CTCAGCCTGTATCAAAGTCACCA 3′) and (5′ CCCACGCCGATGAAGACATA 3′), respectively. The sequence of the forward primer for reference gene *GAPDH* was 5′ CGTCTGCCCTATCAACTTTCG 3′ and that of reverse primer was 5′ CGTTTCTCAGGCTCCCTCT 3′. The specificity of the selected primers was tested using BLAST tool (www.ncbi.nlm.nih.gov/blast).

2.5. Quantitative data analysis of real-time PCR

A SYBR Green real-time PCR was performed on cDNA isolated from MCF-7 cells after treatment with IM. The expression of target gene was quantified by using an ABI 7300 real-time PCR system, according to the following conditions: 95 °C for 10 min, 40 cycles were followed at 95 °C for 15 s and 60 °C for 1 min. Amplification stage was followed by a melting stage at 95 °C for 20 s, 60 °C for 1 min and 95 °C for 20 s. The amplification reaction was carried out at total volume of 20 μL, including 2 μL cDNA sample (100 ng), 1 μL of primers (0.4 μmol/L), 10 μL SYBR Green PCR master mix (2×) and 6 μL double-distilled water. The gene expression was measured by comparative threshold cycle (Ct) values throughout the exponential phase of amplification. During each assay, mCt values were the mean of threshold cycle of Ct values. Relative quantity of target genes (*KAI1/CD82*) was evaluated using comparative Ct method and ΔCt value was determined as the difference between the Ct of target and the Ct of reference gene. The relative quantity of *KAI1/CD82* gene expression compared to *GAPDH* gene was calculated applying the gene dosage ratio formula (Gene dosage ratio = $2^{-\Delta\Delta Ct}$). Where $\Delta\Delta Ct$ = (mCt *CD82* − mCt *GAPDH*)$_{\text{normal sample}}$ − (mCt *CD82* − mCt *GAPDH*)$_{\text{test sample}}$. Gel electrophoresis was used to approve the amplification of PCR products and primer specificity. The fragments of PCR products were separated by electrophoresis in 1.5% agarose gels in 0.5× tetrabromoethane buffer.

2.6. Statistical analysis

The experimental data were performed at least in triplicate and results were expressed as mean ± SEM. *P*-values <0.05

were considered statistically significant and this was assessed using student's *t*-test between sample and control.

3. Results

3.1. MTT assay results

Different concentrations of IM have cytotoxicity effect on MCF-7 cancer cells at 24 h. At concentrations of 1.5, 2.5, 5.0, 10.0, 20.0 and 40.0 μmol/L of IM the viability of MCF-7 was declined to (70.080 ± 0.030)% ($P > 0.05$), (60.480 ± 0.003)% ($P < 0.05$), (34.500 ± 0.008)%, (29.110 ± 0.005)% ($P < 0.01$), (17.520 ± 0.002)% and (9.160 ± 0.007)% ($P < 0.001$), respectively (Figure 1). The IC_{50} of IM on breast cancer MCF-7 cell line was calculated to be 8.96 μmol/L.

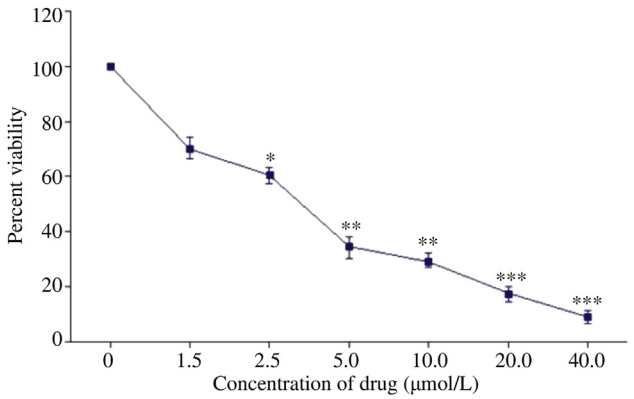

Figure 1. Viability percentage measured by MTT assay on MCF-7 cells exposed to 0, 1.5, 2.5, 5.0, 10.0, 20.0 and 40.0 μmol/L of imatinib mesylate after 24 h.
Data are reported as mean ± SEM from 3 independent experiments. Results were statistically analyzed with a student's *t*-test. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

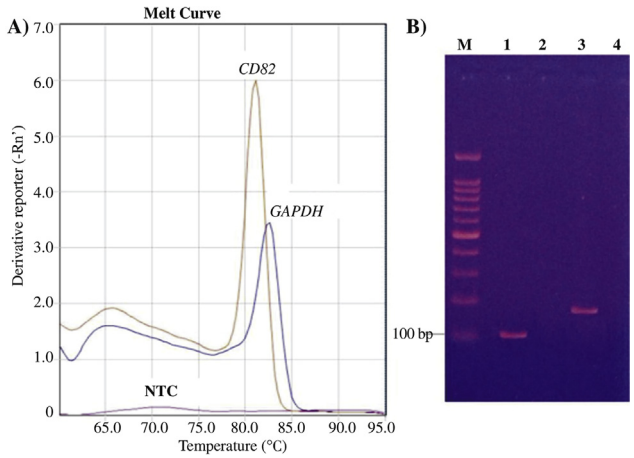

Figure 2. The melting curve of *GAPDH* gene and CD82 gene and gel electrophoresis analysis of the PCR products.
A: The melting curve at 82.4 °C for *GAPDH* gene and 81.1 °C for CD82 gene indicated the specific products that melt at the different temperatures. Flat peak demonstrates non template control (NTC); B: Gel electrophoresis analysis of the PCR products. Lane 1: 102 bp PCR product of *GAPDH* gene. Lane 2: NTC for *GAPDH* gene. Lane 3: 183 bp PCR product of *CD82* gene. Lane 4: NTC for *CD82* gene; M: Molecular Size marker-100 bp ladder.

3.2. Melting curve analysis and gel electrophoresis

The melting curve analysis was evaluated based on ΔRn derivation (derivative of fluorescence over temperature) (y axis) and the temperature at x axis. It was created to document single reaction formation without non-specific products and screen for primer dimmers of desired genes. The melting peaks have been drawn at 81.1 °C for *KAI1/CD82* gene and 82.4 °C *GAPDH* gene as shown in Figure 2A. Moreover, gel electrophoresis analysis of PCR product revealed specific amplification of genes with the expected size (Figure 2B).

3.3. Relative quantification analysis using amplification plots

Evaluation of relative gene expression between treated and untreated samples can be measured by the difference in their Ct values during exponential phase of amplification. The value of mCt for *GAPDH* gene was 17.45 at various concentrations of IM. The mCts values for *KAI1/CD82* gene were 26.96, 26.08, 25.49, and 35.68 at different concentrations of IM ranging from 0, 10.0, 20.0, and 40.0 μmol/L, respectively. The mCt value for untreated samples was scaled as 17.49. The mCt values were scaled as 8.63, 7.99 and 7.92 for 10.0, 20.0, 40.0 mmol/L of IM concentrations. The ΔΔCt values for treated samples at 10.0, 20.0, 40.0 mmol/L IM concentrations were scaled as −0.402, −01.037, and −1.105, respectively. Finally, the gene dosage ratios ($2^{-\Delta\Delta Ct}$) were calculated as 1.322 ± 0.030 ($P > 0.05$), 2.052 ± 0.200 ($P < 0.05$) and 2.151 ± 0.270 ($P < 0.05$) at 10.0, 20.0 and 40.0 μmol/L of drug concentrations (Figure 3).

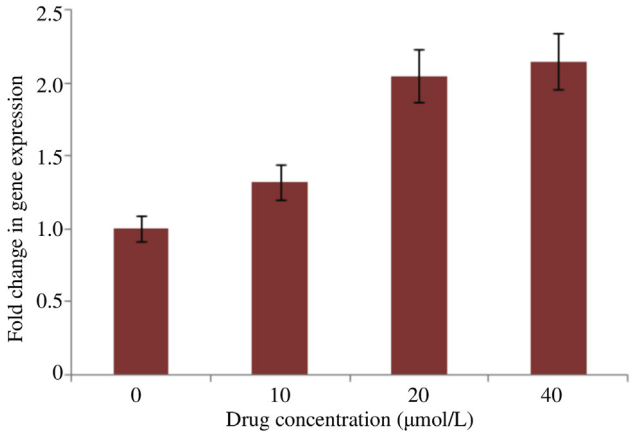

Figure 3. Up regulation of *KAI1/CD82* gene expression in human breast adenocarcinoma (MCF-7) cell line treated with various concentrations of IM.

4. Discussion

Over a million women throughout the world each year are diagnosed with breast cancer which accounts for 25% of all female cancers. The most frequent localization of tumor cells in metastasis such as liver, bone and lungs are dangerous during malignancy of breast cancer. Metastases to bone from breast cancer affect 65–80 percent of patients with progressive malignancy [16].

Imatinib is one of the most widely used tyrosine kinases inhibitors for the treatment of certain cancers that often induce apoptosis and slow progression growth of bone metastases in neoplastic cells which has been recently under examination in clinical trials for malignant gliomas, carcinoid tumor, prostate and ovarian cancer [17]. IM interferes with a specific molecular target included in progression and tumor growth. These targets such as cell-cycle proteins, growth factor receptors, signaling molecules, modulators of apoptosis, and molecules involved in invasion and angiogenesis, which are necessary for development in normal tissues. Nowadays, many researchers showed the *in-vitro* cytotoxicity effects of imatinib on various cancer cell lines [18]. In the present study, we demonstrated that IM exerted a dose-dependent inhibitory effect on the viability of highly metastatic breast adenocarcinoma (MCF-7) cells. Treatment of MCF-7 cells with IM induced the morphological changes which revealed the increase in apoptotic cell population. Tumor metastasis is the most lethal characteristic of a cancer, and it is considered as the most significant contributor to cancer related mortality and morbidity [19]. Tumor metastasis is a very complex process, which involves various stages and several cytophysio logical changes including invasion into the extracellular matrix, migration into bloodstream followed by the extravasate from the circulatory system and initiate colonize distant sites in secondary organ tissues.

Recently, identification of the gene expression during tumor progression has been the great significant of intense method for prognosis and therapy. At the first time, *KAI1/CD82* was discovered as a tumor metastasis suppressor in metastatic prostate cancer cells. *KAI1* was later identified to be metastasis suppressor gene during tumor growth in several solid tumors. It has been shown that *KAI1/CD82* suppresses metastasis by various mechanisms involving inhibition of invasion and motility, induction of apoptosis and senescence in response to extracellular stimuli [20]. Several studies revealed that there are an association between reduced expression of *KAI1/CD82* and increasing metastatic ability in human malignant tumors such as bladder, cervical, ovarian, breast, prostate and hepatocellular carcinoma [21,22]. The interactions of *KAI1/CD82* with several molecules such as epidermal growth factor receptors, chemokines and integrins are likely to play a significant role in cell–cell interaction, signaling and motility of cells, which infer an important role of this gene [23,24]. Other studies showed that *Bax*, *Bad*, *Bcl2*, *PDGF*, *c-kit* genes were differently regulated by imatinib mesylate [18,24,25]. In 2009, Weigel M. *et al*. found that combination of imatinib and vinorelbine induced apoptotic cell death in five human breast adenocarcinoma cells [26]. Fernandes B. *et al*. reported that treating of an animal model with imatinib resulted in 10-fold up regulation of KiSS-1 metastasis suppressor gene, which was then identified as a human metastatic suppressor gene [27]. The consequential finding in our study indicated that imatinib can up-regulate *KAI1/CD82* gene expression in human breast cancer cell line which has not yet been reported. This is the first report depicting significant inhibition of proliferation and increased expression of *KAI1/CD82* gene in breast cancer MCF-7 cells line. Therefore, imatinib remains a promising candidate for the treatment of breast cancer in the future.

In this study we demonstrated that treatment with imatinib for 24 h induces a dose-dependent inhibitory manner on the MCF-7 cells. Also, IM can induce up-regulation of *KAI1/CD82* mRNA levels in MCF-7 in a dose-dependent manner. Based on these results, imatinib probably increases expression of *KAI1/CD82* gene in breast cancer MCF-7 cells. Future subsequent clinical studies on animal tumor models are required to confirm our finding.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This research was financially supported by East Tehran Branch, Islamic Azad University with grant No. 923064. Authors are also thankful to Dr. Ahangari Cohan for his cooperation in technical and support.

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