RESEARCH ARTICLE

Osteopontin b and c isoforms: Molecular Candidates Associated with Leukemic Stem Cell Chemoresistance in Acute Myeloid Leukemia

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Abstract

Despite impressive advances in therapeutic approaches, long-term survival with acute myeloid leukemia (AML) is low as a result of treatment resistance and frequent relapse. Among multitude oncogenic proteins involved in the acquisition of a chemo-resistant phenotype, osteopontin (OPN) recently has attracted marked attention. In spite of the well-defined association between OPN expression and cure rate with solid tumors, there is a scarcity of information on any role of this protein in AML cases. Based on the critical role of OPN in cell survival, it seems reasonable to hypothesize that isoform expression levels may impact on the regulation of apoptosis in AML cells in response to conventional chemotherapeutic drugs and its relation to relapse. To investigate associations between induction of apoptosis and OPN isoform expression, two distinct AML cell lines (KG-1 as a leukemic stem cell model and U937) were treated with chemotherapy drugs, and cell viability and apoptosis were evaluated by MTT and Annexin/PI assay. After determination of appropriate drug doses, mRNA expression levels of OPN isoforms and OPN-related genes were investigated. Our results demonstrated for the first time that acquired up-regulation of OPN-b and c isoforms might prevent conventional chemotherapy regimen-induced apoptosis in AML cells. Moreover, upregulation of OPN-b and c in AML cells appears concurrent with upregulation of AKT/VEGF/CXCR4/STAT3/ IL-6 gene expression. To sum up, this study suggests that OPN-b and c isoforms could be considered as unique beneficial molecular biomarkers associated with leukemic stem cell chemoresistance. Hence, they have potential as molecular candidates for detection of minimal residual disease (MRD) and determination of remission in AML patients. Further evaluation with quantitative real time PCR on patient samples for confirmation appears warranted.

Keywords: Osteopontin- leukemis stem cells- chemoresistance- acute myeloid leukemia

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Introduction

Despite impressive advances in the therapeutic approaches and disease management, still the long-term survival rate of acute myeloid leukemia (AML) is considered to be low as a result of resistance to the conventional chemotherapies and disease relapse (Cogle et al., 2016; Mohammadi et al., 2016a) These phenomena might be related to a small population of resistant malignant cells which are capable of self-renewal and are able to produce large numbers of undifferentiated leukemia cells, known as leukemic stem cell (LSC) (van Rhenen et al., 2007; Pollyea et al., 2014; Shlush et al., 2014; Panah et al., 2017). In the last decades, the increased expression level of specific oncogenes or tumor suppressor genes provides insights into the diagnosis and prognosis of AML (Shahjahani et al., 2015). Among the wide Spectrum of diagnostic molecules, osteopontin (OPN) is one of the novel molecules recognized as being involved in tumorgenesis (Bailly et al., 1997; Rao et al., 2011; Panah et al., 2017)

Osteopontin, also known as secreted phosphoprotein-1 or SPP1, is a glycoprotein which originally secreted by osteoblasts; however, this multifunctional protein is also generated by hematopoietic cells (Anuchapreeda et al., 2006; Liersch et al., 2012; Zahedpanah et al., 2016). A large body of evidence highlighted the importance of OPN in the pathogenesis of different types of solid tumors, such as lung, breast, prostate and colon cancer (Vejda et al., 2005; Rangel et al., 2008). Genetic and biological studies have illustrated that the oncogenic roles of OPN, including induction of limitless cell proliferation, invasion, migration, and growth are regulated through its different isoforms, OPN-a, OPN-b, and OPN-c (Liu et al.,

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2004; Flamant et al., 2005; Nilsson et al., 2005; Mirza et al., 2008; Powell et al., 2009; Zduniak et al., 2015). More recently, it has been suggested that the serum expression level of OPN-b and OPN-c can be regarded as a biomarker for cancer diagnosis. In spite of the well-defined functions of OPN in solid tumors, there is a scarcity of analysis on the role of this protein in hematologic malignancies (Philip et al., 2001; Philip and Kundu, 2003; Rangel et al., 2008; Shevde and Samant, 2014). Our previous studies in monoculture and coculture model demonstrated that OPN appears to be a key gene not only for the detection of MRD but also for the selective elimination of AML-LSCs as a target candidate (Mohammadi et al., 2016b; Mohammadi et al., 2017a). Hence, in the present study, we analyzed the expression of OPN isoforms in both resistants (KG-1) as an LSCs model (Zhang et al., 2010) and sensitive AML cell lines (U937) upon treatment with IDR or DNR in combination with Ara-C as a conventional regiment in AML chemotherapy in the clinic. Moreover, to confirm OPN gene expression data, we investigated the effects of simvastatin and OPN siRNA, as two OPN inhibitors, on the cell proliferation and induction of apoptosis in the indicated cell lines. As far we are aware, this study for the first time showed that OPN-b and c isoforms can be considered as unique beneficial molecular biomarkers which are associated with LSCs chemoresistance. In a nutshell, the findings of current probe suggest these isoforms as substantial molecular candidates for detection of minimal residual disease (MRD) and determination of remission in AML patients.

Materials and Methods

Cell Culture

KG-1 and U937 cell lines (Pasteur Institute, Tehran, Iran) were cultured in RPMI-1640 Medium (Invitrogen, CA, USA) that contained amino acid mixtures, including 4 male L-glutamine and 10% fetal bovine serum (Invitrogen, CA, USA), 1000 units/ml Penicillin, and 100 µg/ml streptomycin. Cells were incubated in humid atmosphere 5% CO2 in 37°C.

Drugs Preparation

Three-drug Daunorubicin (DNR), Cytarabine (Ara-C) and Idarubicin (IDR) (Sigma-Aldrich St. Louis, MO, USA) were dissolved in purified water and make a stoke for treat cell lines.

Microculture Tetrazolium Test (MTT)

The inhibitory effect of three drugs was measured using MTT assay. Each two cell lines were cultured in 96-well plates (SPL Life sciences, Pocheon, Korea) with 5000/100 µL cells per well concentration and then treated with different doses of DNR (0-2 µM), Ara-C (0-20 µM) and IDR (0-4 µM) for 24-72 hours. MTT (Sigma-Aldrich, St. Louis, MO, USA) solution (0.5 mg/ml) was added to each well and the plate was incubated at 37°C in 5% CO_2 atmosphere. DMSO was added to each well and incubated for 4 hours to liquefy the dye crystals. The optical density was read at a wavelength of 570 nm in an ELISA reader.

Annexin / PI Assay

Cells were cultured at 1× 10^6/ml per well in 6-well plates and treated with the cited drug. After 48 hours, each two cell lines were harvested and combined with Annexin/PI according to the manufacturer's instructions (Roche Applied Science, Penzberg, Germany). The stained cells were examined by flow cytometry (Partec, Munich, Germany).

Inhibition of OPN by Simvastatin and Short interfering RNA (siRNA) Transfection

The effect of OPN mRNA expression and also the efficacy of cited drugs in the induction of apoptosis were tested in both cell lines using siRNA and simvastatin (Sigma-Aldrich, St. Louis, MO, USA). siRNA transfection was performed based on the described method (Mohammadi et al., 2016b). Along with siRNA, Simvastatin was used as a natural element for inhibition of OPN too.

RNA Eextraction, cDNA Synthesis and Gene Expression Analysis by Real Time PCR

Total RNA was isolated from the cells using Tripure Isolation Reagent (Roche Applied Science, Peuzberg, Germany) according to manufacturer's instructions. The quantity of RNA samples was assessed spectrophotometrically using Nano drop ND-1000 (Nanodrop Technologies, Wilmington, DE). The cDNA synthesis kit (Takara Bio Inc., Otsu, Japan) was used for generating cDNA from RNA. With using Step One Plus™ (Applied Biosystems, CA, USA) Real Time PCR was completed and we used SYBR Premix Ex Taq technology (Takara Bio Inc., Otsu, Japan). For estimate the relative expression levels we investigated HPRT1 mRNA expression levels and the relative expression was calculated based on the 2−ΔΔCT method (Nikbakht et al., 2017). Nucleotide sequences of the primers were used for Real Time PCR and siRNA sequence (Table.1).

Statistical analysis

All data were presented as means ± SE of triplicate determinants. Data were analyzed using an unpaired two-tailed t-test or χ^2 test. Statistical significance was defined at *P <0.05, **P<0.01, and ***P<0. 001 compared to the corresponding control.

Results

DNR, Ara-C and IDR dose Determination for Molecular Assesment

The cytotoxic effect of three drugs, IDA, DNR, and Ara-C were evaluated in two cell lines KG-1 and U937 to determine the suitable dose for further molecular assessment. In this regard, growth suppressive effects and apoptosis were evaluated by MTT and Annexin/PI assay after treatment with different concentrations of drugs for 24–48 h. Our result showed that IDA, DNR, and Ara-C inhibited cell proliferation with IC50 values of 0.4, 0.5 and 2 µM for U937 cells and 0.8, 0.5 and 4 µM for KG-1 cells, respectively. The results revealed that these chemotherapeutic drugs had a significant cytotoxic effect on both cell linesConsidering dose and time-dependent

Table 1. Nucleotide Sequences of the OPN Specific Sirna and the Primers Used for Real-Time PCR

Gene	Accession number	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size (bp)
OPN-a	NM_001040058.1	ATCTCCTAGCCCCACAGAAT	CATCAGACTGGTGAGAATCATC	208
OPN-b	NM_000582.2	ATCTCCTAGCCCCAGAGAC	AAAATCAGTGACCAGTTCATCAG	209
OPN-c	NM_001040060.1	TGAGGAAAAGCAGAATGCTG	GTCAATGGAGTCCTGGCTGT	155
IL6	NM_000600.4	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG	132
CXCR4	NM_001008540.1	TACACCGAGGAAATGGGCTCA	AGATGATGGAGTAGATGGTGGG	127
STAT3	NM_003150.3	GAGCGGGCCATCCTAAGCACA	TTGGTCTTCAGGTACGGGGCAGC	176
AKT	NM_005163	AGCGACGTGGCTATTGTGAAG	GTACTCCCCTCGTTTGTGCAG	51
β-Catenin	NM_001098209	TACCTCCCAAGTCCTGTATGAG	TGAGCAGCATCAAACTGTGTAG	180
VEGF-A	NM_001316955.1	CTCACCAAGGCCAGCACATAGG	ATCTGGTTCCGAAAACCCTGAG	159
VEGF-C	NM_005429.4	GTCTGTGTCCAGTGTAGATG	AGGTAGCTCGTGCTGGTGTT	360
VEGFR-2 (KDR)	NM_002253.2	GTGACCAACATGGAGTCGTG	CCAGAGATTCCATGCCACTT	660
HPRT	NM_000194	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA	111

siRNA sequence

Name	Sequence	Size (bp)
Sense (5'-3')	GGAAUAUUACUGUGGGAAAdTdT	21
Antisense (5'-3')	UUUCCCACAGUAAUAUUCCdTdT	

manners and IDR and Ara-C were much more effective than DNR.

Since these drugs are used in combination in clinics, the synergistic activities of drugs in combination with each other as well as the viability of treated cells were also assessed 24 and 48 h following the treatments. Taken together, the combination therapy demonstrated significant enhanced effect on these leukemic cells (Figure 1 and Figure 2 A-B).

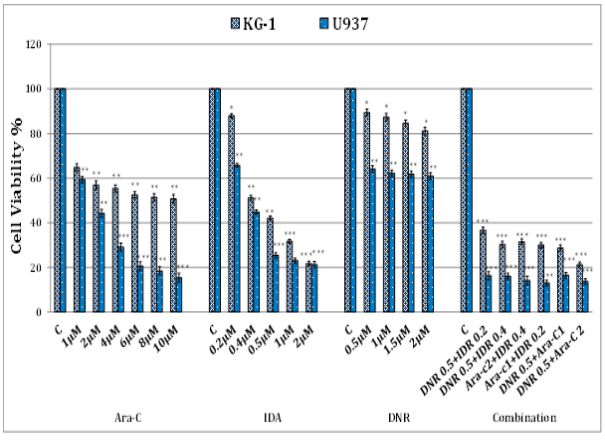

Figure 1. Effects of Ara-C, IDA and DNR with Different Concentration (0-10 Mm) on Cell Viability. The anti- growth effect of cited drugs and their combination were measured by MTT assay following 24-48h in KG-1 and U937 cell lines. IC50 pharmaceutical doses of 0.5μM, 0.4μM and 2μM in KG-1 cells and 0.5μM, 0.2μM and 1μM for Daunorubicin, Idarubicin and Cytarabine in U937 cells were determined respectively. Data indicated that the anti-proliferative effect of these drugs lead to reduction of viability and number of cells in a dose and time dependent manner. Combination of cited drugs were highly effective in inhibition of cell growth and promoting massive apoptosis in both cell lines. Data are mean ± SE of three independent experiments. Statistical significance was defined at *p<0.05, **p<0.01 and ***p<0.001 compared to corresponding control.

The OPN isoforms Expression Level was Increased in Treated AML cells

KG-1 and U937 cells were treated with IDA, DNR, and Ara-C for 48h and then examined for expression of OPN isoforms using Real Time PCR. As shown in Figure 3, the expression level of OPN isoforms was markedly increased in Ara-C-plus DNR and IDA plus DNR-treated KG-1 cells as compared to the untreated cells as well as cells treated

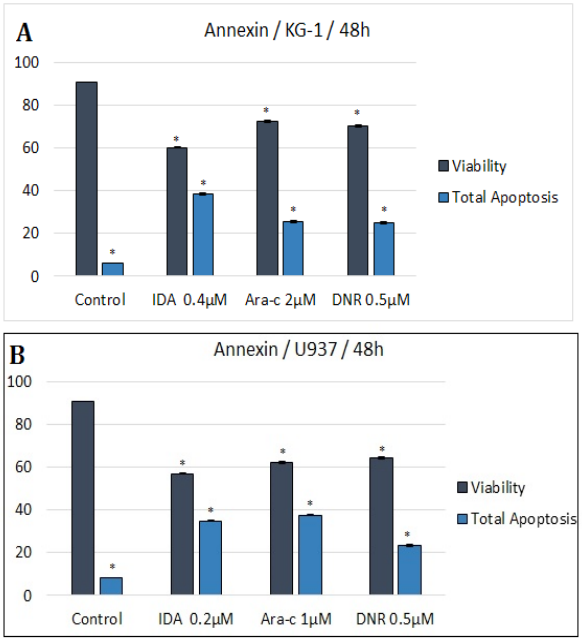

Figure 2. Apoptosis Assay in KG-1(A) and U937 cell lines (B) after 48h. Data are mean ± S.E of three independent experiments. Statistical significance were defined at *P<0.05, **P<0.01 and ***P<0.001compared to corresponding control.

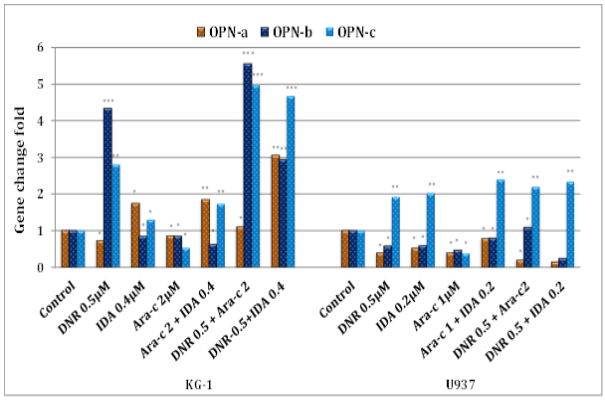

Figure 3. The Effects of Conventional AML Regimen on the Mrna Level of OPN Isoforms in KG-1 and U937 Cells. The effect of this regimen on expression level of OPN was determined by qRT-PCR analysis. Values were normalized using the expression of the housekeeping HPRT. The expression level of OPN isoforms gene expression was markedly increased in treated KG-1 cells compared to the untreated cells and cells that treated with drugs separately. OPN isoforms b and c considered as the predominant isoforms which showed an increasing expression trend in treatment-resistant KG-1 cells as a result of response to treatment, while only OPN-c increased in U937 cell line. Values are given as mean ± S.E of three independent experiments. Statistical significance was defined at *p<0.05, **p<0.01 and ***p<0.001 compared to corresponding control.

with IDA, DNR or Ara-C alone; however, the difference was significant. Separately, Ara-C decreased each three OPN isoforms; however, it increased each three isoforms in KG-1 cells in combination with two other drugs, while only OPN-c increased in U937 cell line (Figure 3).

Simvastatin and OPN Specific siRNA Reduces OPN Gene Expression in AML Cell Lines

In order to prove the effectiveness of drugs on gene expression of OPN isoforms and also to determine whether suppression of these isoforms has an effect on the response to the above-mentioned drugs in our cell lines or not, we used two OPN inhibitors in the present study. We used simvastatin (3-hydroxy- 3- methylglutaryl coenzyme A reductase inhibitor) as a natural OPN inhibitor (Matsuura et al., 2010) and OPN specific siRNA for OPN gene expression inhibition. To determine the effect of simvastatin on OPN expression in vitro, two cell lines were treated with simvastatin (Figure 4A -B) and simvastatin plus three chemotropic drugs for 24-48h (Figure 5). Our result revealed that simvastatin inhibited cell proliferation with IC50 values of 8µM and 16µM for U937 and KG-1 cells, respectively (Figure 4 A-B). Total RNA was then isolated from the cells, and OPN isoforms mRNA expression of AML cell lines was determined by Real Time PCR. According to our results, declared OPN gene isoforms expression was significantly decreased in simvastatin treated groups in KG-1 and U937 cell lines. Likewise, the levels of OPN isoforms mRNA expression were significantly increased in the simvastatin plus drug treated groups in KG-1 cells (*P<0.05) (Figure 6).

Furthermore, we used the specific siRNA against OPN. The obtained data showed that combination of drug with

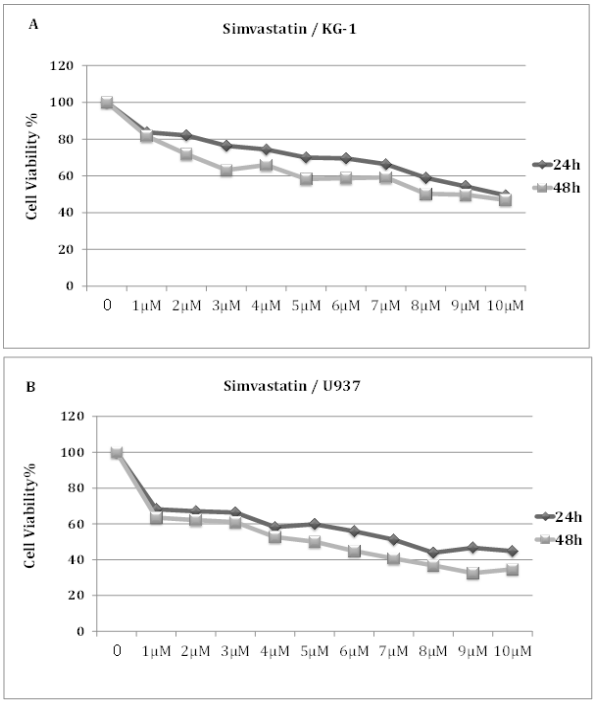

Figure 4. Effects of Simvastatin with Different Concentration (0-10 Mm) On Cell Viability. The anti - growth effect of simvastatin was measured by MTT assay following 24-48h exposed to KG-1 and U937 cell lines. Simvastatin inhibited cell proliferation with IC50 values of 4µM and 8µM for U937 and KG-1 cells respectively. Data indicated that the anti-proliferative effect of this drugs lead to reduction of viability and number of cells in a dose dependent manner.

siRNA can result in a decrease in cell count and viability of cells, more than either drug treatment alone (Figure 7). On the other hand, our results demonstrated that IDR, DNR, and Ara-c can be nullified siRNA and drug -induced apoptosis in AML cell lines by enhancement of OPN

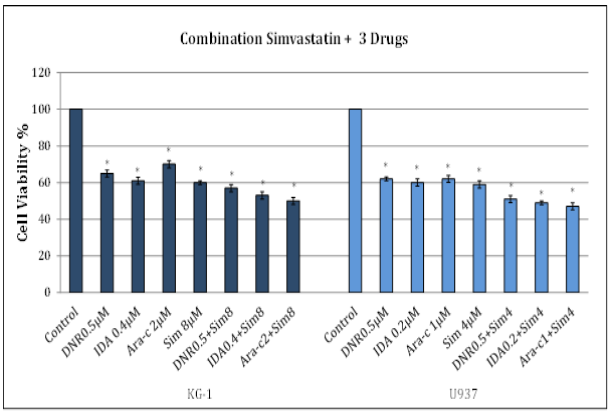

Figure 5. Synergistic Effect Of Simvastatin ((8µm) For KG-1 And (4µm) For U937) And Conventional AML Regimen Drugs On KG-1 And U937 Cells. KG-1 and U937 leukemic cells were treated with drugs alone or in combination for 48h, and then cell count and the metabolic activity were assessed MTT assay. Combined treatments resulted in significant decrease in cell count and viability of cells, more than either compound alone in comparison to control group. Data are mean ± SE of three independent experiments. Statistical significance was defined at *p<0.05 compared to corresponding control.

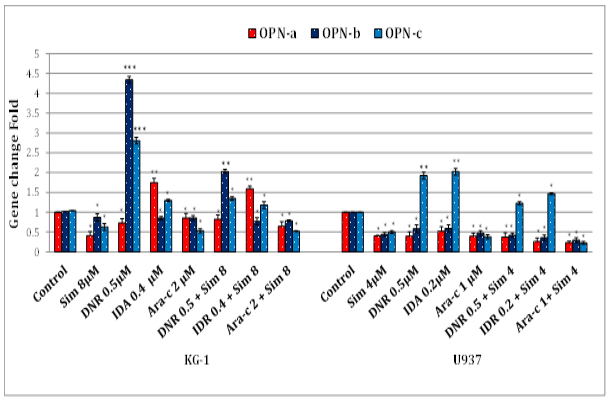

Figure 6. The Effect of Simvastatin on Expression Level of Opn Isoforms was Determined by Qrt-Pcr Analysis. Simvastatin can reduce the OPN isoforms in two cell lines treated with drugs and drugs by enhancement of OPN isoforms gene at mRNA level could be nullified drug -induced apoptosis in AML cell lines. Simvastatin with reduction of the expression of OPN might has a significant contribution to the effectiveness of the drugs. Values were normalized using the expression of the housekeeping HPRT. Values are given as mean ± S.E. of three independent experiments. Statistical significance was defined at *p<0.05, **p<0.01 and ***p<0.001 compared to corresponding control.

isoforms gene at mRNA level (Figure 8).

OPN isoforms Likely Prevent Drug-induced Apoptosis Through AKT/VEGF/STAT3/CXCR4/ IL-6 Molecular Loop

OPN is a secreted protein and may act as both an autocrine/paracrine manner in AML. It may affect several survival signaling pathways and angiogenesis within the cell stimulating cell proliferation and inhibiting cell apoptosis. Therefore, we examined drugs treatment effect on the expression levels of OPN isoforms/AKT/VEGF/

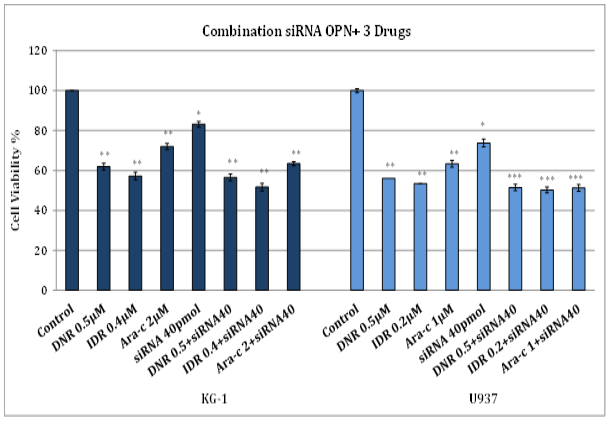

Figure 7. The Effect of Sirna OPN on Cell Viability in KG-1 and U937 Cells. The suppressive effect of siRNA OPN (40pM) for KG-1 and U937 on viability was assessed by MTT assay after 48 h in both cell lines. Combined treatment has result a decrease in cell count and viability of cells, more than either drug treatment alone. The suppression of OPN gene by siRNA increased the susceptibility of two cell lines to apoptosis. Data are mean ± SE of three independent experiments. Statistical significance was defined at *p<0.05 compared to corresponding control.

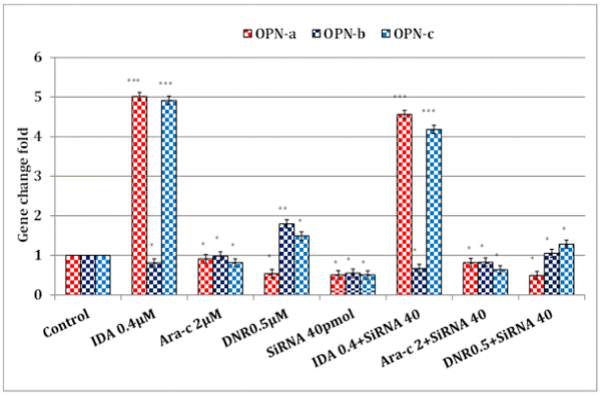

Figure 8. The Effect of Sirna OPN on Expression Level of OPN Isoforms was Determined by Qrt-PCR Analysis. OPN specific siRNA used to knockdown the OPN function and investigated the effect on the OPN isoforms-mediated enhancement of AML cells survival and sensitivity to drugs. The result shows that the suppression of OPN gene by siRNA increased the susceptibility of KG-1 cell line to apoptosis. Moreover, the combination of drugs with siRNA increased the percentage of apoptosis in KG-1 cells. These results suggest that conventional regimen by enhancement of OPN isoforms gene at mRNA level could be nullified siRNA and drug -induced apoptosis in AML cell lines. Values were normalized using the expression of the housekeeping HPRT. Values are given as mean ± S.E. of three independent experiments. Statistical significance was defined at *p<0.05, **p<0.01 and ***p<0.001 compared to corresponding control.

STAT3/CXCR4/IL-6 cellular survival (angiogenesis) pathway by Real-Time PCR to further explore the molecular mechanisms by which OPN isoforms promotes cellular survival under drug treatment.

As shown in Figure 9, the mRNA levels of OPN isoforms concurrent with AKT/VEGF/STAT3/CXCR4/ IL-6 were increased significantly in the treated-KG-1 cells (P<0. 05) after 48 h, as well as U937, treated cells. Furthermore, our result illustrated significant increases in the mRNA levels of AKT/VEGF-A/VEGF-C/STAT3/CXCR4/IL-6 in the 3 drug regimen plus simvastatin-treated KG-1 cells (P<0. 05) in parallel with OPN isoforms enhancement (Figure 9 A-B).

β-catenin gene expression level was decreased after treatment with simvastatin and simvastatin plus drugs in parallel with OPN isoforms in both cell lines. Treatment with simvastatin alone showed a decrease in VEGF-A, β-catenin and AKT gene expression level in both cell lines. On the other hand, VEGF-C, β-catenin, and VEGFR2 (KDR) genes expression level decreased in U937 cell lines after treatment with simvastatin in line with OPN isoforms (Figure-9B). Since, inhibition of OPN isoforms genes was concurrent with a decrease in STAT3, VEGF-C, KDR, and β-catenin genes, it is reasonable to declare a connection between angiogenesis and OPN b and c isoforms and probably these genes are in downstream of OPN b and c. Apparently, OPN b and c are two main OPN isoforms which are involved in angiogenesis by STAT3, VEGF-C, KDR, and β-catenin genes leading to drug resistance and lack of response to the treatment.

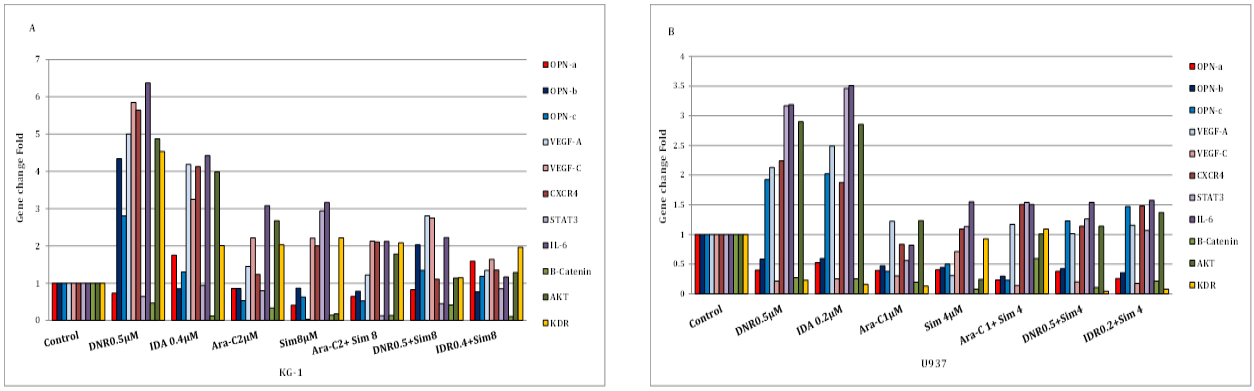

Figure 9. The Effect of OPN Isoforms Inhibitor on VEGF-A, VEGF-C, KDR, CXCR4, STAT3, IL-6 and B-Catenin Gene Expression in KG-1 And U937 Cells. The suppressive effect of OPN inhibitor treatment on KG-1 and U937 and their combination on VEGF-A/VEGF-C/KDR/CXCR4/STAT3/ IL-6/β-Catenin gene expression were assessed after 48 h by qRT-PCR analysis in both KG-1 and U937 cell lines. Values were normalized using the expression of the housekeeping HPRT. The mRNA levels of OPN isoforms/AKT/VEGF/STAT3/CXCR4/IL-6 were increased significantly in the drug plus or without simvastatin-treated KG-1 and U937cells (P<0. 05) after 48 h. In contrast, STAT3 and β-catenin gene expression level were decreased in KG-1 cell lines after drug treatment, while cells in treatment with simvastatin shown a decrease in VEGF-A, β-catenin and AKT gene expression level. VEGF-C, β-catenin and VEGFR2 (KDR) gene expression level were decreased in U937 cell lines after conventional drug treatments.

Discussion

In the recent years, the effective impact of OPN on cancer cell growth and survival is well recognized in various genetic and cancer biology studies (Robertson and Chellaiah, 2010; Mohammadi et al., 2017b). OPN has been found to be expressed in different types of human tumors, including breast, ovarian, lung, and gastric cancers. Moreover, the raised up OPN serum levels have been described CML and AML (Powell et al., 2009); however, the involvement of this multifunctional protein in the pathogenesis of AML is not fully understood. Because, there is an association between OPN expression and tumor cells sensitivity to chemotherapeutic drugs, the researchers' desire to study the role of this protein in various cancers has increased. Based on the critical role of OPN in regulating cell proliferation in HSC niche (Azizidoost et al., 2017), it seems reasonable to hypothesis that the high expression of OPN may participate in disrupting the regulation of cell survival in AML cells (Kwak et al., 2000; Takemoto et al., 2001; Wong et al., 2002; Minoretti et al., 2006). Our previous studies showed that the acquired up-regulation of OPN might prevent curcumin-induced apoptosis and promote enrichment of CD34+/CD38- /CD23+ AML cells as an LSCs surrogate. While activation of the pathway AKT / mTOR / PTEN / β-catenin / NF-κB1 is related to the OPN activity, it is likely to be part of the anti-apoptotic autocrine signaling pathways. (Mohammadi et al., 2016b). Likewise in another study on 2D model, Mohammadi et al demonstrated that upregulation of OPN/CXCL-12/IL-6/ STAT-3 and VCAM-1 in AML cell lines could be a part of the molecular mechanism that induces chemoresistance and enrichment of both LSCs (Mohammadi et al., 2017a).

Herein, based on these data, we aimed to investigate the association between chemotherapeutic drugs-induced apoptosis and OPN isoforms expression in two distinct acute myeloid leukemia cell lines with conventional AML chemotherapy drugs.

The findings showed that following the combined treatment of KG -1 cells with the most well-known chemotherapeutic drugs used in AML treatment (DNR, IDA, and Ara-C), the expression level of all three isoforms of OPN was increased significantly, while maximum elevation observed in OPN isoform c. On the other hand, in U937 cells, only the transcription of OPN isoform c was increased upon treatment with the chemotherapeutic drugs. It is worth to mention that treating both cell lines with the single agent of Ara-c reduced the mRNA expression level of all OPN isoforms; however, the combination of this agent with the other chemotherapeutic drugs had an inductive effect on the gene expression level of OPN isoforms. Our results demonstrated that the mRNA levels of OPN isoforms concurrent with AKT/VEGF/STAT3/CXCR4/ IL-6 were increased significantly in both cell lines. Furthermore, our result illustrated significant increases in the mRNA levels of AKT/VEGF-A/VEGF-C/ STAT3/CXCR4/IL-6 in the 3 drug regimens plus simvastatin-treated KG-1 in parallel with OPN isoforms enhancement. Inline with our finding, Tilli et al., showed that isoform OPN-c stimulates the proliferation of epithelial ovarian IOSE cells through PI3K/AKT signaling pathways indicating the tumorigenic role for this isoform (Tilli et al., 2011). Moreover, other investigations have demonstrated that OPN-c can be considered as a prognostic marker for breast cancer since this isoform takes place in disability of breast cells in the extracellular matrix (He et al., 2006; Mirza et al., 2008). It should be noted that the role of oncogenic OPN-b in gastric cancer cells has been well documented in some research, including Tang et al. (Tang et al., 2013). According to the study conducted by Tang et al., OPN-b strongly induces gastritis cell survival by regulating Bcl-2 apoptotic genes, whilst OPN-c effectively activates metastatic GC activity by increasing the secretion of MMP-2, uPA, and IL-8 (Tang et al., 2013). Based on these notions,

our results suggest that increased expression level of OPN isoforms b and c upon treatment with conventional chemotherapeutic drugs in AML probably contributes to the reduced sensitivity of AML cells to the toxic agents. We also found that treatment of KG-1 cells with either simvastatin or OPN siRNA resulted in the decreased expression level of OPN isoforms, which in turn led to stimulation of apoptotic cell death.

Our results declared a connection between angiogenesis and OPN b and c isoforms. It would seem, OPN b and c are two main OPN isoforms that they have involved in angiogenesis by STAT3, VEGF-C, KDR and β-catenin genes, and leading to drug resistance. Evidence and results from this research are based on the fact that the sensitivity of AML cells to the apoptosis impelled by chemotherapeutic treatments is strongly related to the disorder of STAT-3, β-catenin, VEGF-C and KDR genes. It is important to know that, OPN regulates VEGF expression, and VEGF regulates OPN expression that they control tumor angiogenesis via autocrine, paracrine pathways (Haghi et al., 2017). Regarding the points mentioned above, there is a positive relationship between OPN and VEGF that they can play an important role in growth, development, tumor progression and angiogenesis. (Chakraborty et al., 2008). According to the results of Zhao et al., (2016), IL-6 is also able to induce the VEGF-C production by the JAK-STAT3 signal pathway. Overexpression of CXCR4 is relevant to colorectal cancer (Schimanski et al., 2005), AML (Mohammadi et al., 2017a) and renal cell carcinoma (Wang et al., 2009) and is associated with chemotaxis, invasion, angiogenesis, and cell proliferation.

STAT3 is a key pathway that able to regulate metastasis in cancerous cells (Devarajan and Huang, 2009; Abroun et al., 2015). Also, activation of STAT3 leads to angiogenesis, cell proliferation, and resistance to apoptosis (Yu and Jove, 2004). Over expression of CXCR4/STAT3/STAT3/VEGF-A, and finally, the correlation between CXCR4 and VEGF-A was reported by Wang et al in non -small cell lung cancer. (Wang et al., 2011). Padro et al. presented that VEGFR2 (KDR) commonly express on myeloblasts and VEGF inhibits chemotherapy-induced apoptosis in hematopoietic cells by inducing the anti-apoptotic factor in patients suffering AML (Padro et al., 2002). Our result showed that simvastatin is able to down-regulate STAT3 and β–catenin in KG-1 and VEGF-C, β-catenin, and KDR in U937 cells. In good agreement with our result, Robertson and colleagues reported that OPN promotes cancer cell progression and resistance to apoptosis by activating the AKT/β-Catenin pathway (Robertson and Chellaiah, 2010). KG-1 cells have highly express LSC-associated surface marker CD34 with high clonogenicity (Koeffler et al., 1980; Mohammadi et al., 2016a; Panah et al., 2017). U937 cells have mostly CD34- populations with little clonogenic activity compared with CD34+/CD38- leukemic compartment in KG-1 (Taussig et al., 2010). Hence we hypothesized that U937 cells are depleted of CD34+ LSCs populations with high clonogenicity and it may justify far more β-catenin down-regulation in KG-1 cells as a CD34+ LSCs model (Figure-9A-B). Thus, the most straightforward interpretation of our results is that probably the relationship between acquired up-regulation of OPN isoforms b and c in CD34+ KG-1 cells as well as OPN isoform c in CD34- U937 cells with drug resistance is mediated through regulation of the indicated genes.

In conclusion, taken all together, acquired up-regulation of OPN b and c isoforms in KG-1 probably leads to impedes conventional chemotherapy drugs-induced apoptosis, enrichment of CD34+ AML cells and also its indicative of the significant role of OPN and OPN-related signaling networks for selection and retention of AML-LSCs. Moreover, since LSCs is the main cause of increasing trend of OPN-b, OPN-c, STAT3, VEGF-C, and KDR expression, it can be inferred that OPN isoforms b and c in CD34+ AML and OPN- c in CD34- AML can be regarded as appropriate markers for MRD detection and as a target candidate for the selective elimination of AML-LSCs.

Conflicts of inters

The authors declare no conflicts of interest.

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