Adiponectin enhances Imatinib anti-tumour activity in human chronic myeloid leukaemia cells with serum levels associated with Imatinib efficacy in early chronic phase patients

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Abstract

Objectives: Adiponectin, a functional ligand of adiponectin receptor-1 (AdipoR1) and adiponectin receptor-2 (AdipoR2), has been found to be linked to risk of development of chronic myeloid leukaemia (CML). Imatinib, as its first-line therapy, exhibits striking activity in both chronic and accelerated phases of the condition. However, numerous clinical trials have shown that many patients become refractory or experience relapses. Thus, development of new, hopefully effective Imatinib-based treatment strategies, are still needed.

Materials and methods: Effects of recombinant adiponectin protein, in enhancing Imatinib anti-tumour activities, in K562 and MEG-01 CML cells, were examined *in vitro* and *in vivo*. Forty-eight consecutive newly diagnosed adult patients with Bcr-Abl-positive CML, in the early chronic phase (ECP), were enrolled in the study. Imatinib efficacy, plasma adiponectin levels and their correlations were analysed.

Results: Data presented here indicate that adiponectin enhanced Imatinib efficacy *in vitro* and *in vivo*. Furthermore, this augmented effect was due to inhibition of Bcr-Abl tyrosine kinase activity in an AdipoR1-dependent way, while AdipoR2 was not involved. Most importantly, additional clinical data revealed that adiponectin plasma levels in CML ECP patients, correlated with Imatinib efficacy.

Conclusions: Adiponectin enhanced Imatinib anti-tumour activity in human chronic myeloid leukaemia cells and its serum levels were associated with Imatinib efficacy, in early chronic phase patients.

Introduction

Adiponectin is one of the major adipokines belonging to the complement factor C1q-like protein superfamily, and is composed of an NH₂-terminal collagenous domain and a COOH-terminal globular domain. It has been found to be an important negative regulator of haematopoiesis and is linked to risk of chronic myeloid leukaemia (CML) (1). It has been well established that adiponectin modulates cell proliferation and apoptosis *via* two distinct receptors: adiponectin receptor-1 (AdipoR1) and adiponectin receptor-2 (AdipoR2) (2). Moreover, a recent report has revealed that AdipoR1 expression is significantly higher in CML patients, and this elevation is similar in newly diagnosed, and in Imatinib-treated CML patients (3). Furthermore, adiponectin plasma levels remain extended in CML patients after Imatinib therapy (4). Of note, high body mass index (BMI) has been found to correlate with inferior outcome in CML patients receiving Imatinib, as first-line or second-line treatment (5). Wu and colleagues have demonstrated that adiponectin is able to reverse Imatinib resistance in K562 CML cells *via* AdipoR1 but not *via* AdipoR2 (6). Thus, it is tempting to hypothesize that adiponectin and its functional receptors might be closely related to the function of Imatinib in CML therapy.

CML is a myeloproliferative neoplasm that originates as a haematopoietic stem cell disorder (7) with elevated count of immature white blood cells. The natural history of CML can be described in three clinical phases, following progression from chronic phase to accelerated phase or to rapidly fatal blast crisis, within

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3–5 years. Blood cells retain their ability to undergo terminal differentiation in the chronic phase but not in blast phase (8). CML is usually diagnosed by presence of abnormal Philadelphia (Ph) chromosomes, which result from translocations between long arms of chromosomes 9 and 22. This exchange brings the *Bcr* gene and *Abl* proto-oncogene, together (9). The hybrid gene, *Bcr-Abl*, encodes a fusion protein with tyrosine kinase activity, leading to uncontrolled cell population growth. During progression of CML, the Bcr-Abl oncoprotein is the driving force, which perturbs balance of cell population expansion and cell death of normal haematopoietic cells, and allows malignant properties to develop. Numerous studies have implicated signal transducer and activator of transcription 5 (STAT5) as having an important role in Bcr-Abl-mediated transformation, and disruption of constitutively-activated STAT5 signalling blocks proliferation of CML cells (10–12).

Imatinib (STI571, Gleevec) is commonly the first-line therapy, and exhibits striking activity in chronic and accelerated phases of CML therapy (13). It directly associates with the ATP-binding site Bcr-Abl tyrosine kinase and prevents its kinase activity thus modulating transcription of crucial genes involved in the cell cycle, survival and apoptosis (14,15). Despite high rates of haematological and cytogenetic responses to Imatinib therapy, numerous clinical trials have shown that many patients become refractory to it or experience relapses (16). Management of CML has been effectively and profoundly changed by the introduction of a new generation of tyrosine kinase inhibitors including Nilotinib, Dasatinib and Bosutinib (17), but although methods of management continue to develop rapidly, definitive statements regarding management remain impossible. Various Imatinib combinations have been investigated over recent years; nevertheless, no combination study has reported superior either progression-free survival (PFS) or overall survival (OS) (17). Responses to Imatinib have been defined not only by morphological, cytogenetic and molecular parameters but also by time taken to achieve these responses (16,18). Taken together, given possible benefit of adiponectin in Imatinib efficacy in CML treatment, it is necessary to make further effort to determine whether this hypothesis is viable.

In this study, we evaluated effects of recombinant adiponectin protein in enhancing Imatinib anti-tumour activity in K562 and MEG-01 CML cells. Data presented here indicated that adiponectin enhanced Imatinib efficacy *in vitro* and *in vivo*. Additional data with molecular approaches suggested that this effect was signalled through AdipoR1 but not through AdipoR2, to cause inhibition of Bcr-Abl tyrosine kinase activity. Most importantly, adiponectin serum levels in CML

patients in the early chronic phase (ECP) correlated with Imatinib efficacy.

Materials and methods

Patients and treatments

The study was approved by the Institutional Ethics Committee of the People's Hospital of Liaocheng (Shandong, China), and written informed consent was obtained from every patient. It was undertaken in full accordance with the Declaration of Helsinki, and all further necessary bioethical principles.

Between June 2012 and August 2013, 48 consecutive newly diagnosed adult patients with Bcr-Abl-positive ECP CML received Imatinib (400 mg orally, qd), as first-line therapy. ECP and disease progression were defined in accordance with European LeukemiaNet recommendations for management of chronic myeloid leukaemia (17). Complete haematological response (CHR) was defined as absolute neutrophil count $\geq 1.5 \times 10^9$, platelet count $\geq 100,000 \times 10^9$ and no circulating blasts or extramedullary involvement. Imatinib efficacy was to determine rate of CHR at 3 months, and time to achieve CHR. Peripheral blood samples were obtained and analysed at baseline and twice weekly, for the first 4 weeks, until complete response was achieved and confirmed. They were then taken weekly to week 10, and every 2 weeks after week 10, until medication was stopped.

Enzyme-linked immunosorbent assay (ELISA)

Peripheral venous blood was collected from each patient on the day before initiation of Imatinib treatment. Plasma was collected by centrifugation at $2000 \times g$ for 10 min, and then was stored at -80°C until use. Plasma samples were then thawed to 4°C , centrifuged at $16,000 \times g$ for 15 min at 4°C and adiponectin levels were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Cell lines and culture conditions

Human chronic myelogenous leukaemia K562 and MEG-01 cells (ATCC) were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated foetal bovine serum (Hyclone, Logan, UT, USA). Cells were cultured at 37°C in 5% CO_2 .

Cell viability assay

Cell viability was measured by Cell Counting kit 8 (CCK8) assay. In brief, cells (5×10^4 cells/well) were

plated into 96-well plates containing 100 µL growth medium under indicated treatment at 37 °C in 5% CO₂ for 72 h. Cell viability was assessed using the Cell Counting kit 8 (CCK8; Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Cell population expansion was evaluated by measuring absorbance at 570 nm.

Analysis of apoptosis

Cells were harvested and fixed in 70% ethanol at -20 °C overnight, and washed twice in PBS. They were then labelled for DNA fragmentation using the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling) assay according to the instructions provided by the manufacturer (Roche, Indianapolis, IN, USA). Apoptotic index was calculated after counting a minimum of 8000 events by FACSCalibur (Becton-Dickinson, Mountainview, CA, USA).

Small interfering RNA transfection

Human AdipoR1, AdipoR2 and non-targeting (scrambled) siRNAs were synthesized using the Qiagen method. AdipoR1 and AdipoR2 siRNA sequences were used as previously described (19). Transfection was performed with Transmessenger Transfection Reagent (Qiagen, Shanghai, China) as described by the manufacturer.

Quantitative real-time PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions. 1 µg total RNA was converted to cDNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies). PCR was performed on ABI Prism 7000 apparatus, using corresponding primers and SYBR Green PCR Master Mix (Invitrogen); primer sequences are provided in Table 1. Template cDNA was denatured at 95 °C for 10 min followed by 40 cycles 95 °C for 15 s and 60 °C for 1 min. Quantification data were analysed using ABI Prism 7000 SDS software. Cycle time values were normalized to GAPDH of the same sample. Expression levels of mRNAs were then reported as fold changes versus indicated control.

Enzyme-linked immunosorbent assay (ELISA) for phosphorylated Bcr-Abl and STAT5

Phosphorylated Bcr-Abl and STAT5 were measured using PathScan Phospho-Bcr-Abl (Tyr177) Sandwich ELISA Kit and PathScan Phospho-STAT5 (Tyr694) Sandwich ELISA Kit (Cell Signaling, Beverly, MA, USA) separately, following the manufacturer's instructions. Briefly, cells were lysed in 100 µl per well of 1× cell lysis buffer (kit component). Total soluble protein was quantified using the BCA Protein Assay Kit (Pierce, IL, USA) and the same amount of protein was loaded per well. Each plate was sealed with tape and incubated for 2 h at 37 °C. After four washes in 200 µl 1× wash buffer per well, 100 µl detection antibody reagents were added per well and incubated for 1 h at 37 °C. After four washes in 200 µl 1× wash buffer per well, 100 µl of reconstituted HRP-linked secondary antibody was added per well and incubated for 30 min at 37 °C. Then 100 µl TMB substrate was added. Plates were sealed and incubated for 10 min at 37 °C. Finally, the reaction was terminated by adding 100 µl of STOP solution and plates were read by measuring absorbance at 450 nm.

K562 and MEG-01 xenograft model

BALB/c nu/nu female mice (4–6 weeks of age; Vital River, Beijing, China) were used in this study. All animal experiments were performed in accordance with protocols approved by the Institutional Authority for Laboratory Animal Care of the People's Hospital of Liaocheng.

1.0×10^7 K562 cells or MEG-01 cells in total volume of 0.1 ml were injected subcutaneously into single flanks of the mice, which were then divided randomly into five groups ($n = 8$ in each group) including, (a) an equal volume of saline (buffer control); (b) adiponectin (50 mg/kg); (c) Imatinib (100 mg/kg); (d) Imatinib (100 mg/kg) plus adiponectin (10 mg/kg) and (e) Imatinib (100 mg/kg) plus adiponectin (50 mg/kg). Recombinant human adiponectin was from Peprotech, and was expressed in *Hi-5 Insect* eukaryotic cells according to the manufacturer's instruction. Adiponectins were oligomers, including trimers, hexamers and high molecular

Table 1. Sequence of primers

GENE	Upstream primer (5'-3')	Downstream primer (5'-3')
AdipoR1	CGGTGGAAGTGGCTGAACTG	CCGCACCTCCTCCTCTCTT
AdipoR2	ACGGAGTTGTGACGACTCAC	GCCATCGTCTTGACCTCAC
Bcr-Abl	GGGAGCAGCAGAAGAAGTGT	AAAGGTTGGGGTCATTTTCAC
STAT5	GCCGGCTGTGTATGGTCTAT	AAGTAGTGCCGGACCTCGAT
GAPDH	GAAGGTGAAGGTCTGGAGTC	AGATGGTGATGGGATTTTC

weight (HMW) adiponectin. Endotoxin level of recombinant adiponectin was detected below 0.01 EU per 1 µg by the LAL method.

Treatments began on day 14 (when tumour volumes were ~400 mm³ for K562 cells and ~500 mm³ for MEG-01 cells), for a total of 10 dosages. Imatinib was given orally by gavage and adiponectin was given by intraperitoneal (i.p.) injection once daily. Tumour volumes were calculated using methods described previously (20).

At the end of the study, mice were sacrificed, and recombinant human adiponectin in mouse plasma was measured by ELISA (R&D systems, Minneapolis, MN) according to the manufacturer's protocol. Antibody employed for measurement was specific to human adiponectin and did not cross-react with endogenous mouse adiponectin.

Statistical analysis

Statistical analysis of differences between the treatment modalities was performed using unpaired Student's *t*-testing. *P* < 0.05 was considered to be statistically significant.

Patients were analysed by baseline serum concentrations of adiponectin, low level (5–17.5 µg/ml) or high level (17.5–30 µg/ml). Cut-off 17.5 µg/ml represented median value of serum concentrations of adiponectin of all patients in the study. Time to achieve CHR was measured from date of initiation of Imatinib treatment to date of disease progression. In subgroups categorized by adiponectin serum levels, level of CHR at 3 months was compared using Fisher's exact test. Time to achieve CHR was estimated by employing Kaplan–Meier analysis. Correlations between adiponectin serum levels and CHR at 3 months, or time to achieve CHR, were tested by means of a two-tailed Spearman's correlation coefficient. All reported *P*-values are two-sided. All analyses were performed using GraphPad Prism 5.0 software.

Results

Adiponectin enhanced Imatinib anti-tumour activity in chronic myeloid leukaemia cells in vitro

The effect of adiponectin on viability of K562 cells was accessed by CCK8 assay. As shown in Fig. 1a, adiponectin alone displayed no anti-proliferation trends in either K562 or MEG-01 cells at a variety of dose levels. However, in the presence of Imatinib, adiponectin significantly enhanced Imatinib anti-proliferation of both K562 and MEG-01 cells in a dose-dependent way (Fig. 1b). Interestingly, MEG-01 was more sensitive to

Imatinib under the equivalent dose of adiponectin compared to K562 (Fig. 1b). In addition, analysis of K562 and MEG-01 cell apoptosis, treated by Imatinib combined with various concentrations of adiponectin, corresponded to results of the CCK8 assay. Detectable levels of apoptosis in Imatinib-treated K562 and MEG-01 cells were increased by adiponectin, also dose dependently (Fig. 1c). Coincidentally, apoptotic levels in MEG-01 cells were also markedly higher versus K562 cells, at each adiponectin level. These results prompted us to investigate adiponectin functional receptors in K562 and MEG-01 cells. Interestingly, mRNA expression levels of AdipoR1 in MEG-01 cells were markedly higher than in K562 cells; however, no significant difference was detected in AdipoR2 mRNA expression levels between the two cell lines (Fig. 1d).

Adiponectin signalled through AdipoR1 but not AdipoR2 to enhance Imatinib anti-tumour activity in chronic myeloid leukaemia cells

Based on the above data, AdipoR1 seemed to play a critical role in adiponectin-enhanced Imatinib anti-tumour activity in CML cells. To further investigate functions of AdipoR1 and AdipoR2, specificity and effectiveness of siRNAs for inhibition of transcription in K562 and MEG-01 cells were confirmed using real-time PCR (Fig. 2a,b).

Effective siRNA of AdipoR1 or AdipoR2 was transfected into K562 and MEG-01 cells, then analyses of viability and apoptosis were performed. As predicted, effects of adiponectin-enhanced Imatinib anti-tumour activity in CML cells were attenuated by silencing AdipoR1 but not AdipoR2 in both K562 (Fig. 2c) and MEG-01 cells (Fig. 2d), indicating that enhanced anti-cancer efficacy of adiponectin with Imatinib in CML cells was *via* AdipoR1 but not AdipoR2.

Adiponectin augmented Imatinib-mediated Bcr-Abl inhibition in chronic myeloid leukaemia cells

It is well established that in CML, Bcr-Abl protein leads to enhanced proliferation, resistance to apoptosis and altered adhesion of CML cells, and that Imatinib is able to occupy the ATP-binding pocket of Abl-kinase domain, to exert anti-cancer activity (21,22). Thus, first we tested whether adiponectin would affect mRNA expression levels of Bcr-Abl and its downstream effector signal transducers and activator of transcription 5 (STAT5) in Imatinib-treated CML cells. As shown in Fig. 3a and 3b, mRNA expression levels of Bcr-Abl and STAT5 were not influenced by adiponectin at any dose levels in either K562 or MEG-01 cells. Bcr-Abl

Figure 1. Adiponectin enhanced Imatinib anti-tumour activity in chronic myeloid leukaemia cells *in vitro*. Cell viability was determined by CCK8 assay for K562 and MEG-01 cells treated with (a) indicated concentrations of adiponectin (μg/ml) and (b) indicated concentrations of adiponectin (μg/ml) plus Imatinib (200 nM) for 72 h. (c) Level of apoptosis rate was determined by flow cytometric analysis of TUNEL staining K562 and MEG-01 cells treated with indicated concentrations of adiponectin (μg/ml) in the presence Imatinib (200 nM) for 48 h. (d) Real-time PCR analysis of mRNA expression levels of AdipoR1 and AdipoR2 in K562 and MEG-01 cells. Columns, mean ($n = 4$); bars, SD. NS, not significant. * $P < 0.05$ compared to control in K562 or MEG-01 cells. § $P < 0.05$ for MEG-01 group compared to K562 group. Data are representative of four individual experiments.

phosphorylation was next measured by ELISA. Intriguingly, adiponectin strongly enhanced Imatinib-blocked Bcr-Abl phosphorylation in a dose-dependent manner (Fig. 3c). Furthermore, Bcr-Abl tyrosine kinase activity was determined by assessment of tyrosine phosphorylation of STAT5. As shown in Fig. 3d, adiponectin enhanced Imatinib-mediated STAT5 phosphorylation depletion, also in a dose-dependent manner. More importantly, Imatinib-mediated Bcr-Abl and STAT5 phosphorylation inhibition was abolished by silencing AdipoR1 but not AdipoR2, in both K562 and MEG-01 cells (Fig. 3e,f), indicating that adiponectin augmentation of Imatinib-mediated Bcr-Abl inhibition was *via* AdipoR1, but that AdipoR2 was not involved.

Adiponectin enhanced Imatinib anti-tumour activity in chronic myeloid leukaemia in vivo

To assess enhanced efficacy of adiponectin with Imatinib in CML treatment, two doses of adiponectin (10 and

50 mg/kg *i.p.* qd) with Imatinib efficacy, were determined in mice bearing K562 or MEG-01 tumour xenografts. Results presented in Fig. 4a and 4b demonstrated that adiponectin enhanced Imatinib efficacy in a dose-related way. Anti-tumour activity of adiponectin plus Imatinib was significantly better than that in the group with Imatinib alone; however, adiponectin alone at doses of 50 mg/kg only, showed slight tendency to inhibit the tumour expansion, but no significance was detected. Plasma recombinant human adiponectin of the groups was detected at the end of the study. As shown in Fig. 4c and 4d, plasma exposure in animals treated with adiponectin alone (50 mg/kg) was very close to that of the group undergoing adiponectin (50 mg/kg) plus Imatinib treatment, suggesting that Imatinib did not influence serum recombinant human adiponectin, in the xenograft models. Of note, mice treated with adiponectin (10 or 50 mg/kg) with or without Imatinib, achieved the effective dose here in our *in vitro* study. More importantly, phosphorylated Bcr-Abl and STAT5 in mice

Figure 2. AdipoR1 but not AdipoR2 silencing attenuated adiponectin-enhanced Imatinib anti-tumour activity in chronic myeloid leukaemia cells. (a) Real-time PCR analysis of specificity and effectiveness of siRNAs for inhibition of mRNA expression of AdipoR1 and AdipoR2 in (a) K562 and (b) MEG-01 cells. Effects of siRNAs against AdipoR1 and AdipoR2 on the influence of adiponectin (10 μ g/ml)-enhanced Imatinib anti-tumour activity in both K562 and MEG-01 cells. (c) Cell viability was assessed by CCK8 assay, and (d) Level of apoptosis was determined by FACS analysis of TUNEL staining cells. Columns, mean ($n = 4$); bars, SD. NS, not significant. * $P < 0.05$ compared to control in K562 or MEG-01 cells. § $P < 0.05$ for MEG-01 group compared to K562 group. Data are representative of four individual experiments.

bearing K562 and MEG-01 tumours were dose dependently reduced by adiponectin (Fig. 4e,f), confirming that the mechanism of adiponectin-enhanced Imatinib efficacy might be linked to Bcr-Abl tyrosine kinase activity inhibition.

Taken together, adiponectin-enhanced Imatinib anti-tumour activity in the CML cell line xenograft model, and enhanced anti-tumour activity were due to depletion of Bcr-Abl tyrosine kinase.

Adiponectin plasma levels were associated with Imatinib efficacy in patients with chronic myeloid leukaemia in the early chronic phase

The encouraging *in vitro* and *in vivo* data prompted us to study whether adiponectin levels were associated with Imatinib efficacy, in CML clinical practice. First, we analysed the correlation of adiponectin plasma concentrations, and time to achieve CHR. Surprisingly, adiponectin levels in CML patients were negatively correlated with time to achieve CHR, in our 48 patients with CML in the early chronic phase ($R^2 = 0.5466$; $P < 0.0001$,

Fig. 5a). Recently, high body mass index (BMI) has been reported to correlate with poorer outcome in CML patients undergoing Imatinib therapy (5). However, in our hands, difference in BMIs between the two cohorts was not significant and adiponectin plasma levels in CML patients in ECP still positively correlated with Imatinib efficacy when corrected by BMI ($R^2 = 0.4459$; $P < 0.0001$). Two subsets were then divided according to baseline serum adiponectin concentrations: patients with low adiponectin levels (5–17.5 μ g/ml; $n = 26$) and high adiponectin levels (17.5–30 μ g/ml; $n = 22$). Clinical and laboratory data of those with high adiponectin plasma levels and low adiponectin plasma levels groups are shown in Table 2. There were no significant differences between the two groups in terms of age, sex, BMI, splenomegaly, haematological parameters or Bcr-Abl transcript types. Patients with high circulating adiponectin levels had higher CHR levels at 3 months compared to patients with low adiponectin serum levels (91% versus 77%, $P = 0.004$). Furthermore, median time to achieve CHR in patients with high adiponectin plasma levels was significantly lower than patients with

Figure 3. Adiponectin augmented Imatinib-mediated Bcr-Abl inhibition in chronic myeloid leukaemia cells. (a) Bcr-Abl and (b) STAT5 mRNA expression levels in K562 and MEG-01 cells after treatment with indicated concentrations of adiponectin ($\mu\text{g/ml}$) in the presence of Imatinib (200 nM) for 12 h. ELISA detection of (c) Bcr-Abl phosphorylation and (d) STAT5 phosphorylation in K562 and MEG-01 cells after treatment with indicated concentrations of adiponectin ($\mu\text{g/ml}$) in the presence of Imatinib (200 nM) for 6 h. Effects of siRNAs against AdipoR1 and AdipoR2 on (e) Bcr-Abl phosphorylation and (f) STAT5 phosphorylation in K562 and MEG-01 cells after treatment with adiponectin (10 $\mu\text{g/ml}$) in the presence of Imatinib (200 nM) for 6 h. Columns, mean ($n = 4$); bars, SD. NS, not significant. * $P < 0.05$ compared to control in K562 or MEG-01 cells. § $P < 0.05$ for MEG-01 group compared to K562 group. Data are representative of four individual experiments.

low adiponectin plasma levels (0.6 months versus 1.25 months, $P = 0.0285$, Fig. 5b). Taken together, adiponectin plasma levels in CML patients in ECP correlated positively with Imatinib efficacy.

Discussion

Treatment with Imatinib has undoubtedly revolutionized management and outcome of CML, however, its incapability to eliminate all leukaemia cells in patients, and primary and acquired resistance to Imatinib therapy in some patients, are major problems (23). Although substantial progress has been made towards development of novel drugs, and Imatinib combinations, definitive statements regarding management of CML are still impossible.

To help solve these problems, we examined the therapeutic potential of adiponectin protein at enhancing tumour cell sensitivity to Imatinib. First, we evaluated anti-tumour activity of adiponectin in combination with Imatinib in K562 and MEG-01 CML cells. Our results showed that cell viability/proliferation was significantly reduced by adiponectin in a dose-dependent manner in both K562 and MEG-01 cells, in combination with Imatinib. However, adiponectin itself displayed no anti-proliferation activity in the cells even at high dose

levels, which was consistent with previous observations (24). Correspondingly, analysis of apoptosis also provided equivalent data.

It should also be noted that MEG-01 cells were more sensitive to Imatinib at the same dose of adiponectin compared to K562 cells. In addition, mRNA expression levels of AdipoR1 in MEG-01 cells was markedly higher than in K562 cells. These results prompted us to observe whether adiponectin receptors were involved in adiponectin enhanced Imatinib anti-tumour activity in CML. Our data revealed that the effects of adiponectin-enhanced Imatinib anti-tumour activity was attenuated by silencing AdipoR1 but not AdipoR2, in both K562 and MEG-01 cells, suggesting that AdipoR1 but not AdipoR2 plays a central role in adiponectin signalling in enhanced Imatinib efficacy.

It is well established that Bcr-Abl is characterized by constitutively enhanced tyrosine kinase activity compared to that of normal c-ABL protein, and is known to interfere with a variety of cytoplasmatic and cytoskeletal signalling proteins and cascades, which eventually lead to inhibition of apoptosis (25,26). Very recently, it has been reported that adiponectin is able to reverse Imatinib resistance in K562 cells *via* Bcr-Abl down-regulation (6). However, in our hands, mRNA expression levels of Bcr-Abl remained unchanged by adiponectin

Figure 4. Adiponectin enhanced Imatinib anti-tumour activity in chronic myeloid leukaemia cells *in vivo*. Effect of indicated treatments on volume of (a) K562 xenograft tumours and (b) MEG-01 xenograft tumours ($n = 9$ per group). ELISA detection of recombinant human adiponectin in serum in (c) K562 xenograft models and (d) MEG-01 xenograft models at the end of the study. Columns, mean ($n = 9$); bars, SD. ELISA detection of (e) Bcr-Abl phosphorylation and (f) STAT5 phosphorylation in the K562 xenograft tumours and MEG-01 xenograft tumours after indicated treatments. Columns, mean ($n = 9$); bars, SD. NS, not significant. $*P < 0.05$ compared with control in K562 or MEG-01 cells. $\S P < 0.05$ for MEG-01 group compared with K562 group.

in the presence of Imatinib in both K562 and MEG-01 cells. Inconsistency of results across the two studies might be attributed to long-term exposure to increasing concentrations of Imatinib in K562 cells leading to aberrant high expression of Bcr-Abl, which has also been shown in the study of Wu *et al.* (6). Furthermore, we demonstrated that adiponectin dose dependently augmented Imatinib-mediated inhibition of Bcr-Abl phosphorylation and its downstream molecular STAT5

phosphorylation. These data imply that adiponectin-enhanced Imatinib efficacy might be linked to additive inhibition of Bcr-Abl tyrosine kinase activity with Imatinib. Additionally, this synergistic effect of down-regulation of Bcr-Abl activity was abolished by silencing AdipoR1 but not AdipoR2, in both K562 and MEG-01 cells, indicating that adiponectin-enhanced Imatinib efficacy was *via* AdipoR1, but AdipoR2 was not involved.

Figure 5. Adiponectin plasma levels were associated with Imatinib efficacy in patients with CML in the early chronic phase. (a) Correlations between adiponectin serum levels and time to achieve CHR in patients with CML-ECP ($R^2 = 0.5466$; $P < 0.0001$). (b) Kaplan–Meier estimate of time to achieve CHR in patients with CML-ECP between high adiponectin plasma levels group and low adiponectin plasma levels group (difference between two groups was statistically significant, $P = 0.0285$, log-rank test).

Table 2. Patient characteristics ($N = 48$)

Characteristic	Low adiponectin levels group ($n = 26$)	High adiponectin levels group ($n = 22$)
Male	15 (58)	13 (59)
Age, years	48.1 ± 10.1	51.7 ± 11.5
BMI	25.4 ± 8.1	20.4 ± 8.6
ECOG score, n (%)		
Grade 0–1	24	21
Grade 2	2	1
Splenomegaly, n (%)	9 (35)	7 (32)
Adiponectin levels, $\mu\text{g/ml}$	10.3 ± 2.7	22.6 ± 3.7
Hematologic parameters		
Hemoglobin, g/dl	13.0 ± 4.4	12.5 ± 3.6
White blood cells, $\times 10^3/\mu\text{l}$	20.1 ± 4.5	18.3 ± 3.7
Platelets, $\times 10^3/\mu\text{l}$	353 ± 75	336 ± 101
Bcr-Abl transcript type (%)		
b2a2	47.7	55.9
b3a2	52.3	44.1

Values are number of patients with percentage in parentheses, or mean $\pm$ SD.

ECOG, eastern cooperative oncology group.

NF- κ B, demonstrated downstream of Bcr-Abl, plays an important role in CML, and adiponectin has been reported to suppress the NF- κ B pathway (27,28). Thus, it is tempting to hypothesize that the NF- κ B pathway might be involved in adiponectin enhancement of Imatinib-inhibited Bcr-Abl tyrosine kinase activity in CML cells. Future study was thus required to uncover the mechanism.

In agreement with the *in vitro* data, our *in vivo* study demonstrated that adiponectin dose dependently enhanced Imatinib efficacy in both K562 and MEG-01 xenograft models in mice. Adiponectin combined with Imatinib had a dramatic anti-tumour effect compared to Imatinib alone. Moreover, ELISA assay demonstrated that adiponectin together with Imatinib significantly inhibited phosphorylated Bcr-Abl and STAT5 in the xenograft tumours compared to Imatinib alone. Taken together, adiponectin-enhanced Imatinib efficacy may be due to Bcr-Abl tyrosine kinase activity depletion. Notably, all animals survived adiponectin combined with Imatinib treatment, without any appreciable adverse effects in terms of body weight loss or other signs of toxicity (data not shown) during the treatment; this indicated that the combinations were well tolerated.

Adiponectin has been reported to be linked to risk of CML (1). Expression of AdipoR1 is high in CML patients, and this increase is found in newly diagnosed and Imatinib-treated CML patients (3). Moreover, adiponectin is able to reverse Imatinib resistance in CML cells (6). Indeed, we found that adiponectin plasma levels in ECP CML patients correlated with Imatinib efficacy. In addition, CHR level at 3 months was elevated with high adiponectin circulating levels compared to patients with low adiponectin serum levels. Additionally, median time to achieve CHR in patients with high adiponectin plasma levels was 0.65 months less than in patients with low adiponectin serum levels. Above all, these results indicate that adiponectin serum concentrations may be a potential candidate predictor of clinical benefit for patients CML-ECP treated with Imatinib.

However, in the retrospective design of the current study, some important clinical characteristics had not been recorded, so we are only able to speculate on predictor roles of adiponectin circulating levels in Imatinib efficacy in CML-ECP treatment. Thus, a more comprehensive future study is needed to confirm this fascinating hypothesis. In addition, it has been well established that different isoforms of adiponectin have different activities (29). Whether different isoforms of adiponectin including trimers, hexamers or high molecular weight (HMW) ones have different activities, remains unclear in our current investigation. Future study is needed to elucidate active forms of recombinant human adiponectin in enhancing Imatinib anti-tumour activity in CML.

In conclusion, we have for the first time demonstrated that adiponectin was able to enhance Imatinib anti-tumour activities in CML treatment *in vitro* and *in vivo*. This enhanced effect of adiponectin in Imatinib efficacy was due to depletion of Bcr-Abl tyrosine kinase activity in an AdipoR1-dependent way, while AdipoR2 was not involved. Our data to some degree, have uncovered the mechanism of adiponectin-enhanced Imatinib efficacy that may provide a new and promising therapeutic approach to current CML therapy. More importantly, additional clinical data showed that adiponectin plasma levels in patients with CML in the early chronic phase correlated with Imatinib efficacy, implying that adiponectin plasma levels might be a candidate parameter to predict prognostics of CML-ECP patients under Imatinib therapy.

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Conflict of interest

None.

Reference

- 1 Avcu F, Ural AU, Yilmaz MI, Bingol N, Nevruz O, Caglar K (2006) Association of plasma adiponectin concentrations with chronic lymphocytic leukemia and myeloproliferative diseases. *Int. J. Hematol.* **83**, 254–258.
- 2 Barb D, Williams CJ, Neuwirth AK, Mantzoros CS (2007) Adiponectin in relation to malignancies: a review of existing basic research and clinical evidence. *Am. J. Clin. Nutr.* **86**, s858–s866.
- 3 Ozturk K, Avcu F, Ural AU (2012) Aberrant expressions of leptin and adiponectin receptor isoforms in chronic myeloid leukemia patients. *Cytokine* **57**, 61–67.
- 4 Fitter S, Vandyke K, Gronthos S, Zannettino AC (2012) Suppression of PDGF-induced PI3 kinase activity by Imatinib promotes adipogenesis and adiponectin secretion. *J. Mol. Endocrinol.* **48**, 229–240.
- 5 Breccia M, Loggisci G, Salaroli A, Serrao A, Mancini M, Diverio D, *et al.* (2013) Delayed cytogenetic and major molecular responses associated to increased BMI at baseline in chronic myeloid leukemia patients treated with Imatinib. *Cancer Lett.* **333**, 32–35.
- 6 Wu S, Zheng C, Chen S, Lin B, Chen Y, Zhou W, *et al.* (2015) Adiponectin signals through Adiponectin Receptor 1 to reverse Imatinib resistance in K562 human chronic myeloid leukemia cells. *Biochem. Biophys. Res. Commun.* **456**, 367–372.
- 7 Druker BJ (2002) ST1571 (Gleevec (TM)) as a paradigm for cancer therapy. *Trends Mol. Med.* **8**, S14–S18.
- 8 Kantarjian HM, Dixon D, Keating MJ, Talpaz M, Walters RS, McCredie KB, *et al.* (1988) Characteristics of accelerated disease in chronic myelogenous leukemia. *Cancer* **61**, 1441–1446.
- 9 Rowley JD (1973) Letter: a new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* **243**, 290–293.
- 10 Sonoyama J, Matsumura I, Ezoe S, Satoh Y, Zhang X, Kataoka Y, *et al.* (2002) Functional cooperation among Ras, STAT5, and phosphatidylinositol 3-kinase is required for full oncogenic activities of BCR/ABL in K562 cells. *J. Biol. Chem.* **277**, 8076–8082.
- 11 Huang M, Dorsey JF, Epling-Burnette PK, Nimmanapalli R, Landowski TH, Mora LB, *et al.* (2002) Inhibition of Bcr-Abl kinase activity by PD180970 blocks constitutive activation of Stat5 and growth of CML cells. *Oncogene* **21**, 8804–8816.
- 12 Hantschel O, Warsch W, Eckelhart E, Kaupe I, Grebier F, Wagner KU, *et al.* (2012) BCR-ABL uncouples canonical JAK2-STAT5 signaling in chronic myeloid leukemia. *Nat. Chem. Biol.* **8**, 285–293.
- 13 Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Druker BJ, *et al.* (1996) Inhibition of the abl protein-tyrosine kinase *in vitro* and *in vivo* by a 2-phenylaminopyrimidine derivative. *Cancer Res.* **56**, 100–104.
- 14 Schindler T, Bornmann W, Pellicena P, Miller WT, Clarkson B, Kuriyan J (2000) Structural mechanism for STI-571 inhibition of Abelson tyrosine kinase. *Science* **289**, 1938–1942.
- 15 Gambacorti-Passerini CB, Gunby RH, Piazza R, Galletta A, Rostagno R, Scapozza L (2003) Molecular mechanisms of resistance to Imatinib in Philadelphia-chromosome-positive leukaemias. *Lancet Oncol.* **4**, 75–85.
- 16 Baccarani M, Saglio G, Goldman J, Hochhaus A, Simonsson B, Appelbaum F, *et al.* (2006) Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood* **108**, 1809–1820.
- 17 Baccarani M, Deininger MW, Rosti G, Hochhaus A, Soverini S, Apperley JF, *et al.* (2013) European LeukemiaNet recommendations for the management of chronic myeloid leukemia: 2013. *Blood* **122**, 872–884.
- 18 Baccarani M, Cortes J, Pane F, Niederwieser D, Saglio G, Apperley J, *et al.* (2009) Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. *J. Clin. Oncol.* **27**, 6041–6051.
- 19 Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, *et al.* (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* **423**, 762–769.
- 20 Shi L, Song Q, Zhang Y, Lou Y, Wang Y, Tian L, *et al.* (2010) Potent antitumor activities of recombinant human PDCD5 protein in combination with chemotherapy drugs in K562 cells. *Biochem. Biophys. Res. Commun.* **396**, 224–230.

- 21 Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, *et al.* (1996) Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat. Med.* **2**, 561–566.
- 22 Barnes DJ, Palaiologou D, Panousopoulou E, Schultheis B, Yong AS, Wong A, *et al.* (2005) Bcr-Abl expression levels determine the rate of development of resistance to Imatinib mesylate in chronic myeloid leukemia. *Cancer Res.* **65**, 8912–8919.
- 23 Apperley JF (2007) Part II: management of resistance to Imatinib in chronic myeloid leukaemia. *Lancet Oncol.* **8**, 1116–1128.
- 24 Yokota T, Oritani K, Takahashi I, Ishikawa J, Matsuyama A, Ouchi N, *et al.* (2000) Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* **96**, 1723–1732.
- 25 Warmuth M, Danhauser-Riedl S, Hallek M (1999) Molecular pathogenesis of chronic myeloid leukemia: implications for new therapeutic strategies. *Ann. Hematol.* **78**, 49–64.
- 26 Konopka JB, Watanabe SM, Witte ON (1984) An alteration of the human c-abl protein in K562 leukemia cells unmasks associated tyrosine kinase activity. *Cell* **37**, 1035–1042.
- 27 Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, *et al.* (2000) Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. *Circulation* **102**, 1296–1301.
- 28 Reuther JY, Reuther GW, Cortez D, Pendergast AM, Baldwin AS Jr (1998) A requirement for NF-kappaB activation in Bcr-Abl-mediated transformation. *Genes Dev.* **12**, 968–981.
- 29 Dalamaga M, Diakopoulos KN, Mantzoros CS (2012) The role of adiponectin in cancer: a review of current evidence. *Endocr. Rev.* **33**, 547–594.