Expression analysis of Akirin-2, NFκB-p65 and β-catenin proteins in imatinib resistance of chronic myeloid leukemia

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ABSTRACT

Objective: Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized with the constitutive activation of Bcr-Abl tyrosine kinase which is a target for imatinib, the first-line treatment option for CML. Constitutive activation of NFκB and β-catenin signaling promotes cellular proliferation and survival and resistance to Imatinib therapy in CML. Akirin-2 is a nuclear protein which is required for NFκB-dependent gene expression as a cofactor and has been linked to Wnt/beta-catenin pathway. The purpose of this study is to examine Akirin-2, NFκB and β-catenin in imatinib resistance of CML and to test if any direct physical protein–protein interaction exists between NFκB and both β-catenin and Akirin-2.

Methods: RT–PCR and western blot were performed to determine Akirin-2, NFκB-p65 and β-catenin gene and protein expressions. Co-immunoprecipitation and chromatin immunoprecipitation analysis were carried out to detect the direct physical interactions and binding of NFκB-p65 and β-catenin proteins to MDR1 promoter region, respectively.

Results: β-catenin and NFκB-p65 proteins bound to DNA promoter regions of MDR1 in imatinib-sensitive and resistant CML cells, whereas any direct protein–protein interaction could not be found between NFκB-p65 and Akirin-2 or β-catenin proteins. Nuclear β-catenin and NFκB-p65 levels increased in imatinib resistance. Moreover, increased Akirin-2 protein accumulation in the nucleus was shown for the first time in imatinib resistant CML cells.

Discussion: We show for the first time that Akirin-2 can be a novel biomarker in imatinib resistance. Targeting Akirin-2, NFκB and β-catenin genes may provide an opportunity to overcome imatinib resistance in CML.

KEYWORDS

Akirin-2; NFκB; β-catenin; imatinib; CML; drug resistance; K562; MDR

Introduction

Leukemia is defined as a hematologic malignancy which is characterized with mutations or chromosomal translocations involving transcription factors that cause uncontrolled reproduction in blood cells [1]. Myeloid malignancies consist of chronic stages such as myeloproliferative neoplasms, myelodysplastic syndromes and chronic myeloid leukemia (CML) and acute stages such as acute myeloid leukemia [2]. CML is a malign hematopoietic stem cell disease. In %95 of CML cases, a reciprocal translocation between chromosomes 9 and 22 is seen and BCR-ABL1 gene fusion occurs on the derivative Philadelphia (Ph) chromosome. Ph chromosome is accepted as a definitive marker of CML [3–5].

Imatinib mesylate (Gleevec, STI571, CGP57148B) is an effective therapeutic agent [6]. Imatinib inhibits BCR-ABL1 autophosphorylation and substrate phosphorylation induces, apoptosis and blocks effect of BCR-ABL1 oncprotein [7,8], but resistance to imatinib is the major problem in treatment of this disease. The relationship between β-catenin activation and resistance to tyrosine kinase inhibitors was shown in murine models and primary human CML cells [9–11]. Constitutive activation of NFκB signaling pathway has been observed in several hematologic cancers [12–14] and NFκB-p65 activation was reported in the development of resistance to imatinib [13,15]. In recent years, several NFκB regulators such as Akirin, Nurr 1 and SIRT6 have been determined. Akirin has important functions as a transcriptional cofactor in the embryonic development of Drosophila [16]. In this respect, since NFκB is active in both imatinib sensitive and resistant CML, it is meaningful to investigate Akirin-2, a modulator of NFκB transcriptional activity, in CML. In the literature, very few studies have investigated the role of Akirin-2 in cancer. In one of these studies, the role of Akirin was investigated in basal cell adhesion molecule expressing hepatoma K2 cells and it was found that FB11/Akirin2 is involved in tumorigenicity and metastasis of hepatoma through the downregulation of suppressive oncogenes [17]. Akirin-2 regulates the...
transcription of NFκB dependent genes critically by binding to NFκB via an undefined protein in immune response pathway [18]. It is not known by which mechanism Akirin-2 modulates NFκB transcripational activity. Akirin-2 interacts with Wnt signaling pathway genetically [19] and this interaction is suggested to be a direct interaction with β-catenin or an indirect interaction via adaptor proteins [20].

In this study, we examined the role of Akirin-2 in imatinib-sensitive and resistant CML cells and we checked if any physical interaction exists between Akirin-2 and NFκB-p65 or β-catenin. We found increased nuclear Akirin-2 in K562r cells compared to K562s cells. However, a β-physical interaction was not found between these proteins in both cell lines by Co-immunoprecipitation (Co-ip) assay.

Materials and methods

Chemicals

RPMI 1640 medium (Sigma), fetal bovine serum, penicillin/streptomycin, L-glutamine were obtained from PAA (Pasching, Austria), bisacrylamide, PMSF, APS, glycine, phosphate buffered saline (PBS), anti-β-actin antibody, DMSO were from Sigma (St Louis, MO, U.S.A.), anti-Akirin-2 antibody was from Thermo (Rockford, IL, U.S.A.), anti-p65 and anti-β-catenin antibodies and secondary anti-mouse and anti-rabbit horseradish peroxidase (HRP) antibodies and chromatin immunoprecipitation (ChIP) kit were from Cell Signaling (Danvers, MA, U.S.A.), gel loading dye was from New England Biolabs (Beverly, MA, U.S.A.), primers for Akirin-2 were from Origene (Rockville, MD, U.S.A.), acrylamide, tris base, Hepes, DTT, TEMED, tween 20 from Scharlaeu (Barcelona, Spain), methanol was from LabScan Analytic Science (Gliwice, Poland), bradford reagent was from Bio-Rad (Hemel Hempstead, UK), BenchMark Pre-stained protein ladder and supersignal west femto chemiluminescent substrate was from Thermo (Rockford, IL, U.S.A.). Co-immunoprecipitation and nuclear extraction kit were from Active Motif (Carlsbad, CA, U.S.A.).

Cell culture

K562r (imatinib-resistant) and K562s (imatinib-sensitive) cells were cultured in RPMI 1640 medium (containing 10% heat-inactivated fetal bovine serum, antibiotics (1,000,000 U/ml penicillin and 1 g/ml streptomycin), 2 mM glutamine at 37°C in a humidified 5% CO2–95% air incubator under standard conditions. K562r cells which were resistant to 0.6 μM imatinib were gifted by Prof. Carlo Gambacorti-Passerini from University of Milano-Bicocca, Monza, Italy. Cells were gradually exposed to increasing concentrations of imatinib and their resistance was increased to 5 μM imatinib [21]. Imatinib was provided by Novartis, Pharma AG (Basel, Switzerland). K562r cells were cultured in RPMI medium containing 5 μM imatinib. Imatinib was removed from the media of K562r cells 15 days before performing all experiments to avoid from experimental interference.

Western blot

Akirin-2, NFκB-p65 and β-catenin protein expression differences between K562s and K562r cells were determined with western blot analysis. Cytosolic and nuclear proteins were extracted using nuclear extraction kit (Active Motif) according to the manufacturer’s instructions. The protein concentrations were determined by Bradford Method (BioRad). Equal amounts (20 μg/sample) of protein were separated using %10 and %15 SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. After blocking with milk powder in PBS and then incubating overnight at 4°C with primary antibodies (anti-p65, anti-Akirin-2, anti-β-catenin), membranes were washed with PBS and incubated with appropriate secondary antibodies conjugated to HRP for 1 hour at room temperature then washed with PBST for 2 hours and visualized with ECL agent in a CCD camera (BioRad Chemidoc XRS+).

Co-immunoprecipitation

K562r and K562s cells were lysed and nuclear and cytosolic extracts were obtained with Co-ip kit (Active Motif). Equal amounts of protein extracts (100 μg) were immunoprecipitated with anti-p65 and anti-β-catenin antibodies (Cell Signaling) for 4 hours and magnetic beads (25 μl) were added to the samples for 1-hour incubation in a rotator at +4°C. Immunoprecipitated proteins were then washed four times in Co-ip wash buffer and resuspended in Laemmli sample buffer before loading to SDS polyacrylamide gel for electrophoresis and immunoblotting with anti-Akirin-2, anti-p65 and anti-β-catenin antibodies. K562r cell nuclear lysate was used as a positive control and K562r cell nuclear lysate which did not immunoprecipitate with any antibody at immunoprecipitation level was used as a negative control.

Total RNA extraction and quantitative real-time PCR

mRNA levels of Akirin-2 and β-catenin genes were determined in K562s and K562r cells by qRT-PCR. Total RNA was extracted from cell lines using the High Pure RNA kit (Roche, Mannheim, Germany) according to the manufacturer’s protocols. cDNA was generated from RNA by reverse transcriptase (Transcriptor High Fidelity cDNA Synthesis Kit; Roche). Quantitative real-time RT–PCR was performed using SYBR Green PCR Master Mix (Roche) on the LC480 instrument. mRNA was measured relative to HPRT (hypoxanthine phosphoribosyl
transferred (as an endogenous control). The threshold cycle (Ct) number was detected and used in the comparative Ct method. The relative quantity of the target gene was calculated by the $2^{-\Delta \Delta Ct}$ method. Experiments were performed in biological triplicates. The primers were ATTACAACTTCCA-CAACCT (Forward), CAGACAGATAGCCACCTTAC (Reverse) for $\beta$-catenin and TGACACTGCAAAA-CAATGCA (Forward), GGTCCTTTTCACCAGCAAGCT (Reverse) for HPRT. The primers for Akirin-2 were GATCGGTGTTGACTTCTGATGC (Forward), TCATCC-CAACCTGCGGTAGAGT (Reverse) and purchased from Origene. Cycling conditions were 5 minutes at 95°C, 30 seconds at 95°C, 30 seconds at the annealing temperature and 30 seconds at 72°C. PCR products were separated on 2% agarose gel electrophoresis, stained with ethidium bromide and visualized.

**Chromatin immunoprecipitation**

ChIP assay was performed according to the protocol of the manufacturer of the simple ChiP Enzymatic Chromatin IP Kit (Magnetic Beads, Cell Signaling, Danvers, MA, U.S.A.). Briefly, K562r and K562s cells were incubated in six-well plates for one night and formaldehyde was added to culture medium in a final concentration of 1% for incubation at room temperature. Then, glycine was added at room temperature to stop cross-linking reaction. After washing with PBS, cell fraction was collected and DNAs were sonicated to get 100 bp fragments. Anti-p65 (Cell Signaling) and anti-$\beta$-catenin antibodies (Cell Signaling) bound to magnetic beads were used to select DNA fragments which were exclusively bound to these proteins. Proteins were separated from selected DNA–protein complexes and DNAs were purified. MDR-1 was examined in these DNAs by designing specific primers for these genes promoters and subsequent PCR. Products with bands confirmed binding of NFκB-p65 and $\beta$-catenin proteins to MDR1 gene. Rabbit IgG was used as negative control and Histone H3 was used as positive control.

**Protein expression levels of NFκB-p65, Akirin-2 and $\beta$-catenin proteins in total, cytosolic and nuclear extracts of K562s and K562r cells**

NFκB-p65, Akirin-2 and $\beta$-catenin protein expressions were determined in the total, cytosolic and nuclear extracts of K562s and K562r cells. The results of the western blot experiments showed that NFκB-p65, Akirin-2 and $\beta$-catenin protein expression levels were higher in the nucleus of K562r cells than K562s cells. $\beta$-catenin protein expression was also higher in total and cytosolic extracts of K562r cells (Figure 2).

**Investigation of the interactions between NFκB-p65-Akirin-2 and NFκB-p65-$\beta$-catenin proteins in the nuclear lysates of K562s and K562r cells**

Interactions between NFκB-p65-Akirin-2 and NFκB-p65-$\beta$-catenin were determined by Co-IP in the nucleus of K562s and K562r cells. To check if NFκB p65 and beta-catenin proteins were successfully immunoprecipitated, NFκB-p65 and beta-catenin were also detected in immunoprecipitated cell lysates (Data not shown). It was found that NFκB-p65 protein did not interact with Akirin-2 or $\beta$-catenin in the nuclear lysates of K562s and K562r cells (Figure 3). In order to identify the physical interaction between NFκB-p65 and $\beta$-catenin more precisely, cells were first immunoprecipitated with either NFκB-p65 or $\beta$-catenin and then western blot was performed for detection of NFκB-p65 or $\beta$-catenin proteins in the immunoprecipitated products.

**Detection of NFκB-p65 and $\beta$-catenin binding sites in MDR1 promoter**

Binding of NFκB-p65 and $\beta$-catenin proteins to MDR1 promoter region was investigated with ChIP analysis concurrently. It was not tested whether Akirin-2 had bound to MDR1 promoter since Akirin-2 cannot bind to DNA directly because of the lacking DNA binding

**Results**

**Gene expression levels of Akirin-2 and $\beta$-catenin in K562s and K562r cells**

Akirin-2 and $\beta$-catenin gene expressions were determined with qRT-PCR in K562s and K562r cells. As shown in Figure 1, Akirin-2 and $\beta$-catenin gene expressions were found significantly ($p < 0.05$) higher in K562r cells than K562s cells.

**Statistical analysis**

Results were expressed as means ± SD from at least three independent experiments. Statistical analysis was performed by Student’s t-test. A value of $p < 0.05$ was considered statistically significant.
motif [18]. It was found that NFκB-p65 and β-catenin proteins bound to MDR1 promoter region in both K562s and K562r cells (Figure 4).

Discussion

In the recent years, new participants of NFκB signaling pathway have begun to be found. It was shown that one of these new participants, Akirin-2 had a critical role in innate immunity, embryogenesis as well as in NFκB and Wnt-β-catenin signaling [18,20]. It was reported that Akirin-2 was overexpressed in rat hepatoma cells, glioblastoma cells, pheochromocytoma cells and stimulated cell migration and metastasis. Komiya et al. reported that FBI1/Akirin-2 expression was strong in several tumor cell lines while a strong expression was not observed in non-tumor cells and FBI1/Akirin-2 functioned as an oncogenic protein in LLC1 Lewis lung carcinoma and rat hepatocellular carcinoma K2 cells [22]. However, the role of Akirin-2 in imatinib-sensitive and imatinib-resistant chronic myeloid leukemia has not been elucidated.

In this study, Akirin-2 and β-catenin mRNA expression levels were found to be higher in K562r cells than K562s cells. This finding shows that Akirin-2 and β-catenin may play an important role in the acquisition of drug resistance in CML. Next, we analyzed Akirin-2, NFκB and β-catenin protein expressions in the cytosol and nucleus of CML cells. We found that Akirin-2 protein levels were higher in the nucleus of K562r cells than K562s cells. β-catenin protein expression was also higher in total and cytosolic extracts of K562r cells.
reported that NFkB-p65 inhibition triggered the death of imatinib-sensitive and resistant CML cells including T315I BCR-ABL mutants [25]. β-catenin became activated in myeloid progenitors and translocated to the nucleus in order to regulate the expression of several genes [26]. It was also shown that inhibition of β-catenin genetically or pharmacologically targeted CML stem cells and this might be an effective therapy with imatinib [27]. Collectively, data from our study and recent studies have shown that NFkB and β-catenin signaling has increased in cancer and drug resistance. In addition to all these studies, we detected increased Akirin-2 mRNA and nuclear protein expression in imatinib-resistant CML for the first time. Very few studies have shown the role of Akirin-2 in cancer. Krossa et al. reported that the knock-down of Akirin-2 increased the number of apoptotic cells after temozolomide exposure in glioblastoma cells [28]. It was also shown that FBI1/Akirin2 downregulates suppressive oncogenes and mediates the tumorigenicity and metastasis of hepatoma [17]. On the other hand, its role in leukemia has not been clarified yet.

In this study, physical interaction of Akirin-2 with NFkB-p65 and β-catenin proteins was also investigated in imatinib-sensitive and resistant leukemic cell models. β-catenin was checked for its possible involvement as an adaptor protein for Akirin-2 binding to NFkB-p65 in imatinib-resistant and sensitive cell lines. However, a possible physical interaction between NFkB-p65, β-catenin and Akirin-2 proteins was not detected. Our Co-IP results confirmed that β-catenin was not the adaptor protein responsible for binding NFkB-p65 to Akirin-2. We also examined MDR1 as a common gene target for NFkB-p65 and β-catenin proteins by ChIP. The existence of NFkB-p65 and β-catenin proteins in the same gene region suggested a possible physical interaction between these two proteins. However, such a physical interaction could not be confirmed with Co-IP. NFkB-p65 and β-catenin were found to be bound to their common gene targets and this binding was not changed with imatinib resistance. MDR1 (MDR1/p-glycoprotein) is a glycosylated membrane protein which was originally isolated from tumor cells as a participant of multiple drug resistance mechanism [29]. A study by Bentires-Alj et al. showed that inhibition of NFkB-p65 basal activity downregulated endogenous MDR1 gene expression [23]. In other studies which investigated the link between Wnt pathway and drug resistance, it was reported that activation of Wnt/β-catenin pathway induced upregulation of p-glycoprotein [30] and dual β-catenin and Bcr-Abl inhibition prevented Bcr-Abl kinase-dependent or -independent [31]. In our study, we determined direct binding of NFkB-p65 and β-catenin proteins to the promoter of the MDR1 gene. This is the first study determining the direct binding of β-catenin and NFkB-p65 proteins to MDR1 by ChIP assay.

Even if a direct physical protein–protein interaction between β-catenin, NFkB-p65 and Akirin-2 was not found, they existed on the same promoter of a common gene which indicated their close relationship in both imatinib sensitive and resistant cell lines.

**Conclusion**

In conclusion, these results showed that imatinib-resistant K562 cells exhibited increased nuclear levels of NFkB-p65, β-catenin and Akirin-2 proteins. We suggested that Akirin-2 could be a novel biomarker in imatinib resistance and targeting these proteins could be beneficial to reverse drug resistance of leukemic cells.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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