RESEARCH ARTICLE

Inhibition by Imatinib of Expression of O-glycan-related Glycosyltransferases and Tumor-associated Carbohydrate Antigens in the K562 Human Leukemia Cell Line

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Abstract

Objective: To study changes of tumor associated carbohydrate antigen (TACAs) expression and mRNA levels for tumor associated glycosyltransferases, and assess subcellular localizations of N-acetyl galactosyltransferases (GalNAc-Ts) in the K562 leukemia cell line after imatinib treatment. Methods: RT-PCR was performed to analyze the expression of glycosyltransferases which synthesize O-glycan in tumor-associated carbohydrate antigens (TCTAs). The expression of Tn antigen, T antigen and sialyl T antigen on K562 cell membranes was measured by flow cytometry after treatment with different concentrations of imatinib. Co-localization of GalNAc-Ts and ER (endoplasmic reticulum) was determined by confocal laser scanning microcopy. Results: Transcript expression levels of several glycosyltransferases related to TCTAs were decreased after imatinib (0-0.3μM) treatment. Expression of Tn antigen and T antigen was increased while that of sialyl T antigen was decreased. Co-localization of GalNAc-Ts and ER was reduced by 0.2μM of imatinib. Conclusion: Imatinib inhibited the expression of O-glycan related TACAs and several related glycosyltransferases, while decreasing the co-localization of GalNAc-Ts and ER and normalizing O-glycosylation in the K562 human leukemia cell.

Keywords: Imatinib - K562 - glycosyltransferases - tumor-associated carbohydrate antigens - leukemia cells

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Introduction

Chronic myelogenous leukemia (CML) is a neoplastic disease of the hematopoietic stem cells. It is clinically divided into three phases, chronic phase (CP), accelerated phase (AP), and blast phase (BP) (Faderl et al., 1999). CML is characterized by the Philadelphia (Ph) chromosome, which results from the t (9;22) (q34;q11) balanced reciprocal translocation. The molecular consequence of this translocation is the generation of the BCR-ABL1 oncogene that encodes the chimeric BCR-ABL1 protein with constitutive kinase activity. BCR-ABL1 activates numerous signaling pathways which transform normal cells into oncocytes (Quintás-Cardama et al., 2009). Imatinib is a competitive tyrosine-kinase inhibitor used in the treatment of chronic myelogenous leukemia (CML). Imatinib inhibits 2-phenylamino pyrimidines, which competitively inhibits ATP binding to the ABL kinase domain (Buchdunger et al., 1996), then abrogates the activity of the BCR-ABL oncoprotein through inhibition of BCR-ABL autophosphorylation and substrate phosphorylation leading to suppression of cell proliferation and induction of apoptosis (Deininger et., 1997). K562 cell line was derived from a 53 year old female CML patient of blast phase (Lozzio et al., 1975) The cell line is a typical in vitro model for CML with the BCR-ABL fusion gene. Abnormal expression of O-glycans was reported in leukemia cell lines which could synthesis T, Tn and sialyl-T antigens (Yamada et al., 2012). These simple and short O-glycan structures are called tumor associated carbohydrate antigens (TACAs) (Heimburg-Molinaro et al., 2011). The TACAs are synthesized by the specified glycosyltransferases, where the GalNAc-T2 initializes the synthesis of Tn antigen, C1Gal1 catalyzes Tn to T antigen, ST6GalNAc1 catalyzes Tn to Sialyl-Tn antigen, while ST3Gal1, and ST6GalNAc4 catalyzes T antigen to sialyl-T antigen. Lectins are non-immune origin sugar-binding proteins with different biological functions. Their specificities to discriminate TACAs make lectins good tool to studying the glycan structures.

The expression of O-glycan and tumor metastasis and invasion are closely related. Therefore, to study the expression of glycosyltransferases and TACAs in K562 cells after Imatinib treatment will help to understand the connections among BCR-ABL, glycosyltransferases and O-glycan.

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Table 1. Primer Sets of RT-PCR

Glycogene	Primer sequence (sense, antisense)
GalNAcT2(669bp)	5'-AAGAAAGACCTTCATCACAGCAATGGAGAA -3', 5'-ATCAAAACCGCCCTTCAAGTCAGCA-3'
C1GalT1(224bp)	5'-AGCAGGAGATTCCAGAGATA- 3', 5'-GACGAAGATGATAAACGAGG-3'
ST3Gal1(515bp)	5'-GCTCACCTTCCTCGTGCTCTTCAT-3', 5'-TCCCAACATCAGCTTCAAACCCT-3'
ST6GalNAc1(383bp)	5'-GTCGCTGTGGCTCCAGAAACTC-3', 5'-AGAAGGCGGTAAAGCCGTAGAA-3'
ST6GalNAc2(367bp)	5'-AAGAACTCCCTCGTCTCCTACT-3', 5'-TGATGAATCCATAGGCACTGACC-3'
ST6GalNAc3(401bp)	5'-CCCATTGCTACTAAACTGCT-3', 5'-CATTGCCATCTTTCCTCATA-3'
ST6GalNAc4(256bp)	5'-GCACCCTGCGTGTCGTCTCA-3' , 5'-CGGTTCTTGCCCGTCTCGTC-3'
β-actin(461bp)	5'-GAGCTACGAGCTGCCTGACG-3', 5'-CCTAGAAGCATTTGCGGTGG-3'

Materials and Methods

Cell culture and treatment

K562 cells were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum (Hyclone) and maintained in a humidified tissue culture incubator at 37˚C in a 5% CO_2 atmosphere. Cell concentration was adjusted to 1 × 10^5/mL and Imatinib (Enzo company) solution (formulated as a 1 mM solution and stored at -20 ˚C after filter-sterilization) were added in the cell culture in 0.1μM, 0.2μM and 0.3μM as the three different concentrations. These three groups and one control group are maintained at the same time. After 24 hours of Imatinib treatment, cells were collected and washed with PBS solution prior to the next experiment.

RT-PCR analysis

Total RNA was extracted from the cultured cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. For cDNA synthesis, 2μg total RNA was used as the template in a 25μl reverse transcrip¬tion reaction. To detect GalNAcT2 (669bp), C1GalT1 (224bp), ST3Gal1 (515bp), ST6GalNAc1(383bp), ST6GalNAc2(367bp), ST6GalNAc3 (401bp), ST6GalNAc4 (256bp) mRNA expression, PCR amplification was performed. The PCR primer sequences used were as in the Table 1.

Lectin binding and Flow cytometry analysis

Tn, T, sialyl-T antigens expression level on the K562 cell membrane are identified and quantified by flow cytometry. The biotin labeled lectins were used as primary antibodies and the PE labeled Biotin-streptavidin was used as second antibody. Tn antigen, T antigen and sialyl-T antigen were detected by VVA, PNA and MAL II lectins respectively. Cells were set as 5 × 10^5 per tube and washed with TPBS solution twice. Centrifuged at 1000rpm for 2 mins and resuspended with lectin buffer, then added 500μL MALII, VVA(Vector company), PNA(Sigma) Lectin solutions and incubated 120 mins at 37˚C. Washed with TPBS twice after incubation and added PE labeled Biotin-Streptavidin (Vector company) into tubes with VVA and MAL II lectins, incubation for 60mins at 37˚C again. Washed with TPBS twice and add 380μL PBS buffer (1% BSA) prior to the cytometry analysis for the cell fluorescence intensity.

Colocalization studies by Confocal Laser scanning microscopy

Slides were coated with polylysine and stored at 4˚C. Cells were washed and collected then 20μL cell resuspension was dispensed onto the slides and spread. Used 4% paraformaldehyde solution to fix for 30 mins, then washed twice with PBS and blocked with Carbo-Free solution for 30mins. Washed twice again and incubated with 0.2% Triton-X 100 solution. After blocking with 5% BSA for 1h, cells were stained with primary monoclonal antibody against PDIA4 (Proteintech), the primary antibody were used in a 1:100 dilution and incubated at 4˚C for 10h. Added VVA lectin (Vector company) solution and incubated at 37˚C for 2h. PE labeled Biotin-Streptavidin (Vector company) and FITC labeled secondary antibody were incubated at 37 ˚C for 1h. Nuclei were stained with DAPI (Invitrogen) for 5 min. Staining was analyzed with a confocal laser-scanning microscope (Nikon, Japan).

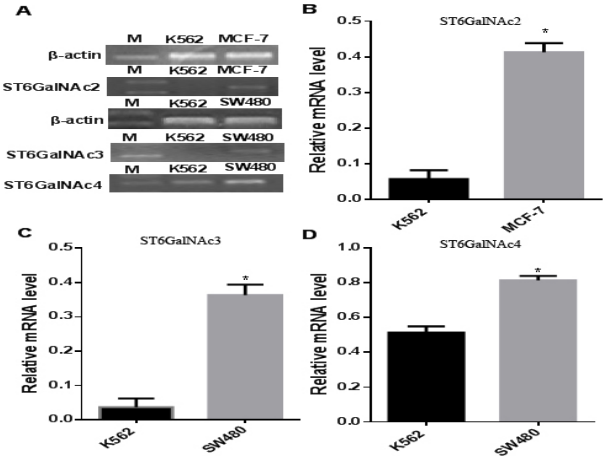

Figure 1. mRNA Expression of ST6GalNAc Family in K562 Cells. A: mRNA expression of ST6GalNAc4, ST6GalNAc3, ST6GalNAc4 in cells K562, MCF-7, SW-480; B-D: ST6GalNAc4, ST6GalNAc3, ST6GalNAc4 mRNA expression level

Statistical analysis

Values are expressed as means ± SD of three independent experiments and a two-sample Student's t-test was performed to compare the treated groups with untreated and control group. Values of P<0.05 were regarded as statistically significant.

Results

Disialyl T antigen synthesis in K562 cells was mainly catalyzed by ST6GalNAc4

There were four members in ST6GalNAc

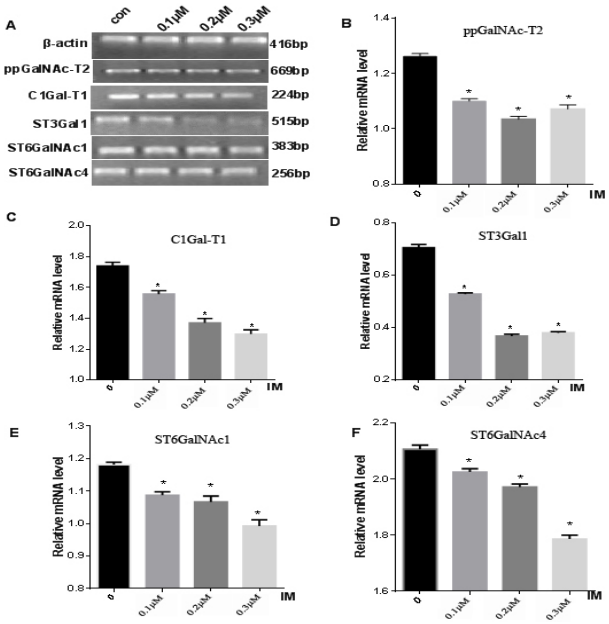

Figure 2. mRNA Expression Level of TACA Associated Glycosyltransferases on O-glycan. A: Control, Imatinib concentration: 0.1μM, 0.2μM, 0.3μM; B-F: ppGalNAc-T2, C1Gal-T1, ST3Gal1, ST6GalNAc1 and ST6GalNAc4 mRNA expression level

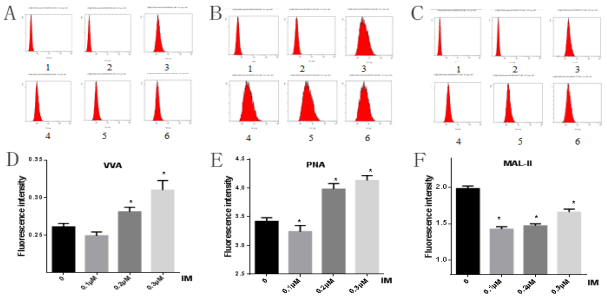

Figure 3. Changes of Tn, T and Sialyl-T Antigen Expression in K562 Cells after Imatinib Treatment. A-C: T, Tn and Sialyl-T antigen expressions were determined by flow cytometry, Tn antigen, T antigen and sialyl-T antigen were detected by VVA, PNA and MAL II lectins respectively (1: Blank Group, 2: Negative Group, 3: Control Group, 4-6:Group 1-3) D-F: histogram of the fluorescence intensity for each group

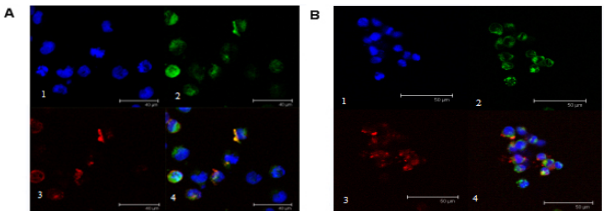

Figure 4. Co-localization of GalNAc-Ts in K562 Cell by Confocal Laser Scanning Microscopy. A: Control B: Group 2(1: DAPI stained nuclei, 2: PDIA4 labeled ER, 3: Lectin VVA and Tn antigen conjugate, 4: Merge of 1-3)

family, ST6GalNAc1, ST6GalNAc2, ST6GalNAc3, ST6GalNAc4. The Sialyl T antigen synthesis DiSialyl T antigen step was catalyzed by one or several enzymes from ST6GalNAc2 to ST6GalNAc4. The RT-PCR revealed that ST6GalNAc4 was highly expressed, in the other hand ST6GalNAc2 and ST6GalNAc3 expression were very low (n=3, $P<0.05$) (Figure 1), which indicated that ST6GalNAc4 mainly catalyzed Sialyl T antigen synthesis Disialyl T antigen in K562 cells .

The mRNA expression of GalNAc-T2, C1 GalT2, ST3Gal1, ST6GalNAc1, ST6GalNAc4 in K562 cells after Imatinib treatment

The mRNA levels of some glycosyltransferases synthesizing O-glycan and associated with TACAs in tumors such as GalNAc-T2, C1 GalT2, ST3Gal1, ST6GalNAc1, ST6GalNAc4 were reduced in different levels after Imatinib stimulation. The GalNAc-T2 and ST6GalNAc1 expressions were reduced by low dose of Imatinib stimulation and increased when the doses were increasing, but still lower than the control. With the increase of imatinib concentration, the expression of three glycosyltransferases C1GalT1, ST3Gal1 and ST6GalNAc4 were sequentially reduced (n=3, $P<0.05$) (Figure 2).

Tn, T, and Sialyl-T antigens expression level were altered in K562 cells after Imatinib treatment

After incubated with Imatinib at 0.1μM for 24hr, T and Tn antigens on cell membrane were lower than control group (n=3, $P<0.05$). When the Imatinib concentration were increased, T and Tn antigens increased accordingly (n=3, $P<0.05$). T and Tn antigens expressions were higher than the control group after incubated with Imatinib at 0.2μM and 0.3μM for 24hr. Sialyl-T antigen was lower than the control group after Imatinib treatment for 24hr, and increased with the increasing of Imatinib concentration, but the level was still lower than that of the control group at 0.3μM (Figure 3).

Co-localization of GalNAc-Ts in K562 cell after Imatinib treatment

K562 cells were treated with 0.2uM imatinib after 24 hours of incubation, as shown in Figure 4, the overlap degree of the red fluorescence and green fluorescence decreased which means the co-localization degree between Tn antigen and ER decreased. The reasons may be GalNAc-Ts were refluxed from the ER to the Golgi partially by COP-I vesicles, which could increase Tn antigen synthesis in the Golgi.

Discussion

Glycosylation is one of the common post-translational modifications of the protein. Variation in oligosaccharide structures on proteins play an important role in the biological processes of cell growth and differentiation, recognition, immune response and signaling (Hakomori et al., 1985; Hakomori et al., 1996). In the process of tumor genesis, cell surface glycosylation changes, expressing high level of a tumor associated carbohydrate antigens, high sialylation and insufficient phosphorylation. These abnormal glycosylations are closely related to the adhesion, invasion, metastasis and poor prognosis of tumor biological behavior (Brockhausen et al., 2006).

The most important characteristic of CML(Chronic Myelogenous Leukemia) is high tyrosine kinase activity. The current study suggests that SFKs (Src-family kinases) have been implicated in BCR-ABL signaling

(Danhauser-Riedl et al., 1996; Warmuth et al., 1997). In turn, the activity of BCR-ABL can be enhanced through SFK-mediated Phosphorylation (Lionberger et al., 2000; Boggon et al., 2004). In addition, the study shows that SFKs also regulate subcellular localization of GalNAc-Ts which initiate the glycosyltransferases of O-glycosylation. In normal cells, GalNAc-Ts mainly localized in Golgi and initiate the synthesis of O-glycans, however, in tumor cells, SFKs activity enhanced, GalNAc-Ts were redistributed from Golgi to ER by COP-I vesicles and brought back to the Golgi after O-glycan synthesizing in ER (Gill et al., 2010). The relocalization of GalNAc-Ts to the ER promotes a significant increase in the density of GalNAc modification which formed high density of GalNAc-Ser/Thr structure (Tn antigen) and blocked the binding between other glycosyltransferases and Tn antigen, which would result in synthesis of a higher proportion of short O-glycans. Therefore, tumor cells showed increased density of short glycans (Gerken et al., 1998; Brockhausen et al., 2009). These high expressions of short O-glycan structure such as Tn antigen and sialyl-T antigen were observed in CML cell line K562 (Yamada et al., 2012), and explained why BCR-ABL kinase leads to highly expression of TACAs in CML cell line K562. Therefore, to inhibit the activity of BCR-ABL kinase in K562 cells with Imatinib might give a pathway to inhibit SFks activity indirectly and normalize O-glycan synthesis.

The results showed that GalNAc-T2 and ST6GALNAc1 were down-regulated when the Imatinib concentration was low, and increased after Imatinib concentration raised up, but still lower than the control group even at highest concentration of Imatinib. On the contrary, C1GalT1, ST3Gal1 and ST6GALNAc4 were decreased when the concentration of Imatinib increased. Imatinib could inhibit BCR-ABL kinase activity specifically. It is speculated that signaling molecules in BCR-ABL kinase signaling pathway might be inhibited by Imatinib which consequentially down-regulate the expression of these glycosyltransferases expression.

The flow cytometry results showed that Tn antigen expression was slightly increased, which is more related to the down regulation of ST6GalNAc1 and ST3Gal-T1 than to the decrease of C1Gal-T1. In O-glycan synthesis, ST6GalNAc1 and ST3Gal-T1 catalyze T antigen to synthesize sialyl T antigen, and disialyl T antigen in further (Reis et al., 2010). It may be induced by Imatinib, which inhibited ST6GalNAc1, ST6GalNAc4 and ST3Gal1 expression, decreased Sialyl-Tn and Sialyl-T antigen synthesis from Tn and T antigen. Tn and T antigen sialylation were insufficient and exposed, so the expression level of Tn and T antigen elevated. Sialyl-T antigen expression was increased, but still lower than the control group even after incubation with 0.3μM Imatinib. Our previous study found the ppGalNAc-T2 was related to the proliferation, adhesion and invasion of leukemia cell (Liu et al., 2011). Other scientists revealed that aberrant O-glycosylation can reduce the stability of anti-adhesion molecule dysadherin and up-regulate E-cadherin to enhance the adhesion (Hitomi et al., 2003). In this study, Sialyl T antigen was highly expressed in K562 cells, which is related to the high expression of TCTA related Glycosyltransferases. It is inferred that the inhibition of CML cell adhesion and invasion in patients after Imatinib treatment may achieved through suppression of TCTA related Glycosyltransferases.

The subcellular localization studies indicated the alteration of GalNAc-Ts after Imatinib stimulation. It was observed that the co-localization degree between GalNAc-Ts and ER decreased by confocal laser scanning microscopy indicating that after the activity of BCR-ABL kinase in K562 cells was inhibited by Imatinib, the SFKs activity was inhibited indirectly. This could prohibit GalNAc-Ts to reflux to ER through COP-I trafficking, decrease the GalNAc-Ser/Thr structure and normalize the O-glycan synthesis. The flow cytometry showed that the TACAs expression was down regulated after incubation with Imanitib. It is indicated that BCR-ABL kinase might impact the initialization of O-glycan synthesis in K562 cells through enhancing SFKs kinase activity and up-regulate the expression of short O-glycan structure. The relationship between BCR-ABL kinase and O-glycan regulation on cell membrane needs further investigation.

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