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Folypolyglutamate Synthetase Gene Transcription is Regulated by a Multiprotein Complex that Binds the TEL-AML1 Fusion in Acute Lymphoblastic Leukemia

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Abstract

Acute Lymphoblastic Leukemia (ALL) non-random fusions influence clinical outcome and alter the accumulation of MTX-PGs *in vivo*. Analysis of primary ALL samples uncovered subtype-specific patterns of folate gene expression. Using an FPGS-luciferase reporter gene assay, we determined that E2A-PBX1 and TEL-AML1 expression decreased FPGS transcription. ChIP assays uncovered HDAC1, AML1, mSin3A, E2F, and Rb interactions with the FPGS promoter region. We demonstrate that FPGS expression is epigenetically regulated through binding of selected ALL fusions to a multiprotein complex, which also controls the cell cycle dependence of FPGS expression. This study provides insights into the pharmacogenomics of MTX in ALL subtypes.

Keywords

FPGS; TEL-AML1; Rb/E2F; Chromatin remodeling; ALL; leukemia; HDAC1; methotrexate

1. Introduction

Childhood Acute Lymphoblastic Leukemia (ALL) is the most common malignancy affecting children and adolescents [1]. ALL is a heterogeneous disease with a range of unique clinical,

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genetic, molecular, immunologic and pharmacologic characteristics. Gene expression profiling studies have identified at least seven distinct subtypes in which six of them corresponded to ALL subgroups previously clustered around recurrent chromosomal abnormalities and non-random translocations [2]. These chromosomal aberrations define important biological pathways in ALL, and have been recognized to be prognostic and consequently are currently used to determine risk-stratified therapy [3,4]. Fusion proteins encoded by non-random translocations in ALL are strong enhancers or repressors of gene transcription, in particular for tissue-specific genes [5]. They lead to the formation of aberrant transcription complexes that disrupt normal programs for hematopoietic cell differentiation, and can regulate biochemical pathways responsible for drug metabolism [5].

The antifolate methotrexate (MTX) continues to be a universal component of most successful ALL regimens [4]. MTX's cytotoxicity is largely dependent on the intracellular accumulation of the pharmacologically active metabolites, long-chain MTX polyglutamates (MTX-PGs). The synthesis of MTX-PGs is catalyzed by the enzyme folylpoly- γ -glutamate synthetase (FPGS) [6]. The net pharmacologic effect of MTX polyglutamation is prolonged intracellular retention and increased DHFR inhibition, plus inhibition of additional key folate related enzymes such as thymidylate synthase (TS) and aminoimidazolecarboxamide ribonucleotide (AICAR) transformylase [6]. Polyglutamation of novel antifolates such as pemetrexed also results in pharmacological profiles that lead to potentiation of its cytotoxicity, and therefore determine its clinical activity [7,8]. We have previously shown that FPGS mRNA and protein expression are controlled in a lineage-specific (Bp-ALL vs. T-ALL) and proliferation dependent manner within cells of lymphoid origin, and that a correlation exists between higher *in vivo* intracellular accumulation of MTX-PGs and improved outcome in patients with ALL [9,10]. Transcription of the human FPGS gene is regulated by a TATA-less promoter driven by a set of 8 concatameric Sp1 sites spaced within a 150 bp region upstream of exon 1 [11], and by binding of the NFY (Y-box) transcription factor [12]. In gene array studies, the expression of FPGS and other folate-related genes was found to differ among ALL subtypes [13]. Others have reported that expression of the most common ALL fusion protein, TEL-AML1, and DNA ploidy are associated with distinct patterns of accumulation of MTX-PG in primary samples from B-precursor ALL [14].

Recently we reported that treatment with histone deacetylase inhibitors (HDACi) resulted in increased FPGS expression and higher intracellular accumulation of long chain MTX-PG₃₋₇ in relevant ALL models [15]. In addition, we found that the combination of HDACi (NaBu and SAHA) plus MTX exhibited synergistic induction of apoptosis in these cell models. We also established that increased FPGS expression in cells treated with HDACi resulted from epigenetic mechanisms involving histone modifications and chromatin remodeling through the NFY-B and Sp1 mediated recruitment of HDAC1 to the FPGS promoter [15].

In the present study, we investigated the mechanism by which selected non-random chromosomal translocations and cell cycle progression alter FPGS expression and subsequently influence the accumulation of MTX-PGs in ALL lymphoblasts. For this purpose, the effect of distinct non-random genomic translocations on FPGS, ABCC1, ABCG2, DHFR, GGH, SLC19A1, and TS mRNA expression were analyzed in 109 primary Bp-ALL patient samples and representative cell line models using real-time qRT-PCR. Using ChIP assays we determined that HDAC1, AML1, mSin3A, E2F, and Rb proteins associate with the active chromatin structure of the FPGS promoter region to regulate its expression. We demonstrate that both TEL-AML1 and E2A-PBX1 down-regulated FPGS gene transcription through association with this multiprotein complex, and that this complex also influences FPGS expression throughout lymphoblast cell cycle progression. On this basis, we propose a model for the epigenetic regulation of FPGS mRNA expression by a multiprotein complex in cells expressing selected ALL fusions, which also regulates its expression during cell cycle

progression. Our data demonstrate for the first time a causal relationship between selected gene fusions, down-regulation of FPGS expression and provides insights into the mechanism that determines the cell cycle dependence of FPGS expression in lymphoid cells.

2. Materials and methods

2.1 Patient samples and cell lines

One hundred and nine Bp-ALL patient samples (26 hyperdiploid (>50 chromosomes), 29 expressing t(1;19)/E2A-PBX1, 26 expressing t(12;21)/TEL-AML1, and 28 with double Trisomy 4/10) were provided by the Children's Oncology Group ALL Cell Bank after NCI-CTEP approval (Protocol AALL05B2) for analysis of relevant folate related genes. Additionally, representative cell line models were examined. The human leukemia cell lines NALM6 (Bp-ALL) and REH (Bp-ALL t(12;21)) were obtained from the DSMZ (Germany) and the American Type Culture Collection (ATCC, Rockville, MD), respectively. RCH-ACV (Bp-ALL t(1;19)) and AUXB1 cell lines were kindly provided by Dr. Stephen Hunger (The Children's Hospital, Denver, CO) and Dr. Richard Moran (VCU, Richmond, VA), respectively. The AUXB1 cell line is a Chinese hamster ovary-derived cell line containing a point mutation in the FPGS gene resulting in auxotrophy to adenosine, thymidine, and glycine [16]. NALM6, RCH-ACV and REH cell lines were grown in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS at 37°C and 5% CO₂. The AUXB1 cell line was grown in MEM- α medium supplemented with 10% fetal bovine serum (Mediatech, Inc., Herndon, VA).

2.2 Plasmids

The pGL1374 plasmid containing the FPGS gene promoter::luciferase reporter fusion was constructed as described previously [17]. The pCMVKJ7 plasmid expressing E2A-PBX1 gene fusion was generously provided by Dr. Stephen Hunger (University of Colorado, Denver, CO). The pMSCV-I-YFP-TEL-AML1 plasmid expressing TEL-AML1 gene fusion was generously provided by Dr. Gerard Grosveld (St. Jude Children's Research Hospital, Memphis, TN). To construct pE2A-PBX1 (CMV promoter driven E2A-PBX1 expression), a 2787 bp *EcoRI*-*XbaI* fragment was excised from pCMVKJ7 and inserted into the pCI-neo vector containing the neomycin selectable marker (Promega, Madison, WI). Similarly, to construct pTEL-AML1 (CMV promoter driven TEL-AML1 expression), a 2394 bp *EcoRI* fragment from pMSCV-I-YFP-TEL-AML1 was cloned into the pCI-neo vector digested with *EcoRI*. Genetic orientations were screened using restriction enzyme digestions and identity of each plasmid was confirmed by nucleotide sequencing.

2.3 Transfection and luciferase reporter gene assays

The AUXB1 FPGS null mutant cell line was transfected by nucleofection (program U-23) as described in the Amaxa nucleofection protocol (Lonza, Basel, Switzerland). Briefly, 5×10^6 cells were resuspended in 100 μ L of solution T and mixed with 2 μ g of plasmid pGL1374 (6.2 kbp) and equimolar concentration of pCMVKJ7 (7.5 kbp) or pMSCV-I-YFP-TEL-AML (8.9 kbp), and the internal reference pCMV- β vector (7.2 kbp) (CMV IE promoter driven β -galactosidase expression, BD Biosciences). As control, the empty vector pCDNA6/V5-His/C (5.1 kbp) (Invitrogen) was used. Following transfection, cells were cultured for 24 hrs at 37°C/5% CO₂ in 6-well plates pre-treated with poly L-lysine. Then, cells were harvested, washed twice with cold PBS 1X and aliquot for total RNA and whole cell extractions. Luciferase and β -galactosidase activities were assayed using the dual-light reporter gene assay system (Tropix, Inc., Bedford, MA). Level of E2A-PBX1 and TEL-AML1 mRNA expression was determined by qRT-PCR as described by Lanza et al [18] and Gabert et al [19], respectively. Data were normalized to β -actin mRNA expression [15]. All real-time qRT-PCR reactions (SYBR green) were performed using the BIO-RAD iCycler iQ system (Bio-Rad) [15].

2.4 Generation of NALM6 stable cell lines expressing E2A-PBX1 or TEL-AML1

To generate NALM6 stable cell lines expressing E2A-PBX1 or TEL-AML1, 5×10^6 NALM6 cells were transfected with 5 μ g of pE2A-PBX1 or pTEL-AML1 plasmids using nucleofection (solution V; program G016) and cultured for 24 hrs in RPMI-1640 drug free medium supplemented with 10% FBS. The following day, transfectants were selected with 600 μ g/ml Geneticin (G418 sulfate, Invitrogen, Carlsbad, CA) and cultured for 6-8 weeks in presence of Geneticin. Transfectants were analyzed for E2A-PBX1 or TEL-AML1 mRNA expression using qRT-PCR as previously described [18,19]. Results were normalized to β -actin mRNA expression [15]. All real-time qRT-PCR reactions (SYBR green) were performed using the BIO-RAD iCycler iQ system (Bio-Rad, Hercules, CA) [15]. Level of E2A-PBX1 or TEL-AML1 protein expression was determined by Western blots using anti-human PBX1 (556348; BD Biosciences, San Jose, CA) and anti-RUNX1 (N-20/sc-8563, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody, respectively.

2.5 RNA isolation and TaqMan real-time qRT-PCR

Total RNA was isolated from cells using the RNeasy kit (Qiagen, Inc., Valencia, CA). First strand cDNA synthesis was performed using AMV reverse transcriptase (Promega). Level of FPGS, gamma-glutamyl hydrolase (GGH), solute carrier family 19/folate transporter (SLC19A1, formerly RFC), ATP-binding cassette subfamily C (ABCC1), ATP-binding cassette subfamily G (ABCG2), thymidylate synthetase (TS), and dihydrofolate reductase (DHFR) mRNA expression was measured by real-time quantitative qRT-PCR using primers and TaqMan probes (IDT) as described [20,21,22]. For all genes, we used 6-carboxyfluorescein (FAM) as reporter dye. Relative level of mRNA gene expression was normalized using a hexachlorofluorescein (HEX) labeled β -actin probe. Each of the probes was quenched using Black Hole Quencher-1 at its 3'-end. All TaqMan qRT-PCR assays were performed using the iQ Supermix on the iCycler iQ5 system (BIO-RAD). Each experiment was performed at least two times in tetraplicate.

2.6 Flow Cytometry Assessment of Cell Cycle

CCRF-CEM and NALM6 cells were blocked in S-phase using hydroxyurea [23]. Briefly, NALM6 and CCRF-CEM cells were treated with hydroxyurea (10 μ g/ml) for 24 hrs and cultured in drug-free media for 4 hrs and 24h at 37°C to synchronize cells in S-phase and G1 phase, respectively. Routinely, 70-80% and 60-70% of cells arrested in S-phase and G-phase, respectively, were assayed for total RNA extraction, whole cell extraction, and cell cycle analysis. The cellular DNA content was assessed by flow cytometry as previously described [24]. Cells (2.0×10^6) were washed twice with PBS, resuspended in 70% ethanol and fixed overnight at 4°C. Subsequently, cells were washed twice with PBS and suspended in 0.5 ml of PI/RNase solution (BD Biosciences/Pharmingen) and cell cycle progression was determined by flow cytometry (BD Biosciences FACSCalibur flow cytometer). Level of FPGS mRNA expression was determined using real-time qRT-PCR as described previously [15]. Level of FPGS and acetyl-Histone H3 expression was determined by Western blots using anti-FPGS (sc-98479, Santa Cruz Biotechnology, Inc.) and anti-acetyl-Histone H3 (06-599, Millipore, Billerica, MA) antibody, respectively.

2.7 Chromatin Immunoprecipitation Assay (ChIP)

Chromatin immunoprecipitation (ChIP) assays were performed using the EZ ChIP Assay Kit (Millipore). Briefly, chromatin extracted from either NALM6 or REH cells was cross-linked with 1% formaldehyde and DNA sheared to the lengths of 200–1500 bp using sonication. Precleared chromatin aliquots were immunoprecipitated overnight at 4°C with 10 μ g of polyclonal antibodies against acetyl-Histone H3 (06-599; Millipore), AML1 (PC284; EMD Biosciences, San Diego, CA), HDAC1 (ab7028; abcam, Cambridge, MA), HDAC1 (sc-6298),

E2F-1 (sc-193), RUNX1 (sc-8563), NFYB (sc-13045), Rb (sc-50), mSin3A (sc-994), or normal rabbit IgG (sc-2025) (Santa Cruz Biotechnology, Inc.). Complexes were recovered using protein A-agarose beads, washed and eluted according to kit instructions. Following reverse cross-linking with 0.2M NaCl, chromatin DNA was digested with proteinase K and purified using a ChIP DNA Clean and Concentrator kit (Zymo Research Corp., Orange, CA). Eluted DNA was subjected to PCR using primer set 19679F (5'-CCCGGAGCGTACACTCATAAA) and 20109R (5'-CGCAGGCCCCACGTGTCGTC) to amplify a 449bp fragment of the FPGS promoter region. The PCR products were resolved on 2.5% agarose gel and visualized with ethidium bromide staining. ChIP assays were performed in duplicate using independent chromatin preparations.

3. Results

3.1 Level of FPGS and other folate-related gene mRNA expression in ALL subtypes

Based on the reported differences in clinical response to antimetabolite-based chemotherapy regimens in distinct ALL phenotypes [25,26,14,27], we hypothesized that chromosomal abnormalities and non-random translocations present in ALL subtypes alter the expression of FPGS and other folate-related genes, and lead to differences in MTX metabolism. To test this hypothesis, we examined mRNA levels of MTX transport genes (ABCC1, ABCG2, and SLC19A1), MTX polyglutamation genes (FPGS and GGH) and MTX target genes (DHFR and TS) using TaqMan real-time qRT-PCR in 109 Bp-ALL primary samples expressing the t(1,19)/E2A-PBX1 (n=29), t(12,21)/TEL-AML1 (n=26), double trisomies 4/10 (n=28), or hyperdiploidy (n=26) phenotype. As shown in Figure 1, we found significantly lower level of FPGS mRNA expression in cells expressing E2A-PBX1 and TEL-AML1 fusions (Global F-test: $p=0.0004$; Kruskal-Wallis: $p=0.0002$), but no significant differences for GGH mRNA expression (responsible for hydrolysis of the polyglutamate tail in reduced folates and classical antifolates) (Global F-test: $p=0.5927$; Kruskal-Wallis: $p=0.3731$). These results indicate that once inside lymphoblasts, accumulation of MTX-PG is dependent on FPGS expression in ALL subtypes. Consistent with these findings, reduced levels of FPGS mRNA expression were also detected in the representative cell lines models RCH-ACV and REH expressing E2A-PBX1 and TEL-AML1, respectively, when compared to control (NALM6 cells) (Figure 3A). These data suggest that E2A-PBX1 and TEL-AML1 alter FPGS mRNA expression and may lead to decrease metabolism to MTX-PG. In addition, we found significantly lower levels of mRNA expression of the transporter SLC19A1 in cells expressing E2A-PBX1 and TEL-AML1 fusions. Similar results were obtained for ABCC1 in primary ALL cells harboring E2A-PBX1. In contrast, significantly higher level of ABCG2 mRNA expression was detected in cells expressing TEL-AML1. No significant difference in mRNA expression was observed in the MTX target genes DHFR and TS. Our data are consistent with a previous report in a different cohort of patients using gene expression arrays which correlated expression of folate-related genes and *in vivo* accumulation of MTX-PG in Bp-ALL subtypes [13].

3.2 E2A-PBX1 and TEL-AML1 expression downregulate FPGS transcription at the promoter level

Based on our data from primary patient samples, we then determined whether TEL-AML1 or E2A-PBX1 fusions are responsible for decreased FPGS mRNA expression by constructing NALM6 stable cell lines using a pCI-neo vector expressing E2A-PBX1 or TEL-AML1 and evaluated the level of FPGS mRNA expression. Using qRT-PCR and Western blots, we first confirmed that NALM6/E2A-PBX1 and NALM6/TEL-AML1 stable transfectants expressed E2A-PBX1 and TEL-AML1 mRNA transcripts and protein (Figures 2A and 2B, respectively). In these stable transfectants expressing either E2A-PBX1 or TEL-AML1, the level of FPGS mRNA expression was decreased by approximately 30% compared to untransfected control

cells, indicating that the expression of these fusion proteins resulted in the down regulation of FPGS mRNA transcripts (Figure 2C).

To determine the mechanism leading to decreased FPGS mRNA expression in cells expressing the TEL-AML1 or E2A-PBX1 fusions, we examined FPGS promoter activity using an FPGS-luciferase reporter gene assay. The FPGS null mutant AUXB1 cell model was triple transfected with constructs expressing FPGS-luciferase, CMV- β gal to normalize the level of luciferase activity, and either pE2A-PBX1 or pTEL-AML1. As control, AUXB1 cells were mock transfected with the empty vector pCDNA6/V5-His/C. Following transfection, cells were assayed for luciferase and β -galactosidase activities, and levels of TEL-AML1 and E2A-PBX1 mRNA and protein expression were determined using real-time qRT-PCR and Western blot (data not shown). As shown in Figure 3B, expression of E2A-PBX1 and TEL-AML1, resulted in 60% decrease in the level of FPGS-luciferase activity compared to mock transfected control ($p < 0.005$ for E2A-PBX1; $p < 0.01$ for TEL-AML1), indicating that expression of both translocations led to down regulation of FPGS promoter activity.

3.3 AML1, HDAC1, and mSin3A interact directly with the FPGS promoter

Recently, our laboratory reported that HDAC inhibitors (NaBu and SAHA) upregulated FPGS mRNA expression in ALL cell lines, with higher level of induction detected in cells expressing TEL-AML1 and BCR-ABL fusions [15]. These findings suggested that FPGS expression is regulated by epigenetic mechanisms involving histone modifications and chromatin remodeling. Both AML1 and TEL proteins have been shown to associate with mSin3A to regulate gene expression [28,29,30]. It has also been reported that TEL-AML1 represses gene expression through its interaction with HDAC1, NCoR and mSin3A [31,29,32,33,28]. Based on the data presented herein demonstrating that TEL-AML1 negatively regulates FPGS promoter activity and our previous findings that HDAC1 associates with the FPGS promoter region, we used ChIP assays to investigate whether TEL-AML1 and mSin3A also associate with the FPGS promoter region in its native chromatin structure. Chromatin extracted from TEL-AML1 positive REH cells was immunoprecipitated with HDAC1, mSin3A and AML1 antibodies, and DNA subjected to PCR amplification using primers designed to amplify a 449 bp specific fragment within the FPGS promoter region. As shown in Figure 4, the 449 bp PCR product was detected in TEL-AML1 positive REH cell's chromatin immunoprecipitated with either HDAC1, mSin3A or AML1 antibodies, but not in the controls with normal rabbit IgG or without antibody. These findings indicate that HDAC1, mSin3A and AML1 associate with the native chromatin structure surrounding the FPGS promoter region in REH cells expressing TEL-AML1. These data are therefore consistent with TEL-AML1, HDAC1 and mSin3A (and possibly others) associating around the FPGS promoter region as members of a multiprotein complex that regulates FPGS expression.

3.4 FPGS expression is epigenetically regulated during cell cycle progression

In lymphoid cells, our laboratory and others have shown that regulation of FPGS expression is both lineage-specific and proliferation dependent [34,9,35]. To investigate these mechanisms we determined the cell cycle dependence of FPGS expression and examined histone modifications and chromatin remodeling as epigenetic mechanisms regulating FPGS expression. To first investigate the extent in which FPGS expression is regulated during cell cycle progression, we determined the level of FPGS mRNA expression in NALM6 (Bp-ALL) cells during cell cycle checkpoints using real-time qRT-PCR. As shown in Figures 5A and 5B, when NALM6 cells were synchronized to G1 and S phases, the relative level of FPGS mRNA and protein expression was 2.9- and 1.9-fold higher in G1-phase than in S-phase, respectively. Similar level of FPGS mRNA expression was detected in CCRF-CEM cells arrested in S-phase and synchronized to G1-phase (1.4-fold higher in G1 vs. S-phase; data not shown). The upregulation of FPGS expression likely occurs in G1 phase prior to progression into S-phase

allows cells to accommodate the increased folate and nucleoside requirements needed for DNA replication in anticipation of cellular division. Similar cell cycle related changes have been reported for two other key folate pathway enzymes, DHFR and TS [36,37,38].

To examine the role of histone modification and chromatin remodeling in FPGS cell cycle regulation, we first determined the level of histone H3 acetylation in the native chromatin structure of the FPGS promoter region from NALM6 cells treated with the HDACi, SAHA. Chromatin was extracted from NALM6 cells treated with $\pm$ SAHA, immunoprecipitated with anti-acetyl-histone H3 antibody, and DNA subjected to PCR amplification using primers designed to amplify a 449 bp specific fragment within the FPGS promoter region. As shown in Figure 5C, treatment with SAHA increased the ratio of ChIP/input DNA compared to controls, indicating that increased histone acetylation is responsible for the induction of FPGS expression in NALM6 cells treated with HDACi. Similar increase of histone H3 acetylation was detected in the native structure of the FPGS promoter region in REH cells treated with $\pm$ SAHA (data not shown).

Under our experimental conditions, when chromatin was extracted from NALM6 cells arrested in G1 and S phases and immunoprecipitated with anti-acetyl-histone H3 antibody, no significant differences in the level of acetyl-histone H3 was detected in the native chromatin structure of the FPGS promoter region in G1 vs. S-phases (data not shown). In contrast, we detected in Western blots higher level of acetylated histone H3 protein in NALM6 cells arrested in G1- compared to S-phase which correlated with the higher level of FPGS mRNA and protein expression detected in G1- vs. S-phase (Figure 5D). These data suggest that epigenetic mechanisms involving histone modifications and chromatin remodeling regulate FPGS expression during cell cycle progression.

3.5 E2F1 and Rb proteins interact with the FPGS promoter region during cell cycle progression

The cell cycle dependence of FPGS expression in ALL cell models prompted us to consider known cell cycle regulators as potential members of the putative multiprotein complex suggested by our data. It is known that the Rb/E2F pathway, which recruits HDAC to E2F and targets promoters to repress transcription, controls multiple cell-cycle regulated genes, including DHFR [38,39]. Based on the observed induction of FPGS expression in the G1 phase, we determined whether the E2F and Rb proteins regulate FPGS expression by altering chromatin structure during cell cycle progression using ChIP assays. NALM6 cell's chromatin was immunoprecipitated with HDAC1, E2F, Rb, and NFYB antibodies, and DNA subjected to PCR amplification using primers designed to amplify a 449 bp fragment of the FPGS promoter region. The NFYB antibody was used as a positive control of a transcription factor capable of binding to the active chromatin structure of the FPGS promoter region [15]. As shown in Figure 6, the 449 bp PCR product was detected in NALM6 cell's chromatin immunoprecipitated with E2F, HDAC1, NFYB and Rb antibody but not with normal rabbit IgG or without antibody. We interpret these results to indicate that E2F and Rb participate in a multiprotein complex that assembles around the native chromatin structure of the FPGS promoter to regulate FPGS expression during cell cycle progression.

Taken together, our data suggest that a multiprotein complex that assembles at the FPGS promoter region alters FPGS expression by regulating constitutive promoter activity and mRNA expression during cell cycle progression. Putative members of this multiprotein complex include AML1, E2F, HDAC1, mSin3A, NFYB, Rb, and Sp1, and this complex also associates with distinct ALL fusion proteins to determine FPGS expression in specific ALL phenotypes (Figure 7). The mechanism by which this multiprotein complex leads to altered FPGS expression in lymphoid cells involves changes in histone acetylation status in the FPGS promoter region.

4. Discussion

The control of FPGS expression in lymphoid cells is lineage-specific and proliferation dependent [35,34,9]. In patients with ALL, higher levels of FPGS mRNA, protein and enzymatic activity are observed in B-lineage compared to T-lineage ALL, and these differences correlate with their sensitivity to antifolates such as MTX [10,26,25,9]. Previous studies from our laboratory determined that the described minimal promoter region is sufficient for maximal transcriptional activity of the FPGS gene in T-ALL but not in Bp-ALL [12]. We now present data that implicates a multiprotein complex that assembles at the FPGS promoter region and epigenetically regulates FPGS gene expression in ALL subtypes and during cell cycle progression. Non-random translocations encoding fusion proteins which characterize most ALL subtypes, are known to alter gene transcription, induce changes in specific molecular signaling pathways, and consequently have the ability to influence response to existing therapies [5]. Herein, we used primary ALL cells and cell line models to establish that non-random fusions alter folate-related gene expression, supporting the postulate that distinct patterns of folate-related gene expression are present in Bp-ALL subtypes. The latter results are consistent with a previous report using Affimetrix gene arrays [13], but unlike gene expression profiling which uses statistical algorithms for data analysis which can impact the interpretation of the results, our expression data generated using real-time quantitative RT-PCR in fully characterized primary patient samples, provides unambiguous evidence that the expression of distinct ALL fusion proteins influence folate-related gene expression and likely result in distinct pharmacological profiles with respect to MTX metabolism. Further, our data demonstrates for the first time that the mechanism by which selected fusions alter the pharmacology of MTX polyglutamation is by binding to a multiprotein complex assembled at the FPGS promoter region which determines its constitutive expression. Understanding the effect of these fusions on drug metabolism in general, and on the molecular pharmacology of MTX in particular, has already begun to provide a biological basis to rationally design novel combination strategies for ALL [15,40].

In the course of our investigation, we determined that selected fusion proteins, such as TEL-AML1 and E2A-PBX1, downregulate FPGS promoter activity and lead to lower mRNA expression and enzymatic activity. Using Co-immunoprecipitation and ChIP assays, we demonstrated that TEL-AML1 decreased FPGS transcription by recruiting co-repressors (mSin3A, Rb) and HDAC1 to the FPGS promoter region. Further, we provided evidence that TEL-AML1, mSin3A, and HDAC1 interact with the chromatin structure of the FPGS promoter region to regulate its expression. Our data strongly suggest that the association of TEL-AML1 with mSin3A and HDAC1 represses FPGS transcription and leads to lower level of MTX-PGs intracellular accumulation described in these phenotypes [14]. Our findings are consistent with previous data indicating that both TEL and TEL-AML1 interact with mSin3A and N-CoR to regulate gene expression [30,28,41]. As we recently demonstrated, treatment with an HDAC1 inhibitor (such as SAHA or NaBu) will inhibit HDAC1 activity and disrupt interactions between Sp1, NFY and HDAC1 leading to changes in histone acetylation and FPGS promoter activation [15]. Indeed, the combination of SAHA and MTX *in vitro* led to synergistic induction of cell death in ALL cell models. These findings support the clinical use of HDACi plus antifolates subject to polyglutamation by FPGS as a synergistic combination in high risk ALL, or as a strategy to overcome resistance due to impaired polyglutamation.

FPGS activity was linked to cellular proliferation in transformed cells and during organ development in a rat embryo model [34]. The proliferation dependence of FPGS expression during periods of rapid cell division correlates with the higher requirement for reduced folates as a result of increased DNA synthesis in proliferating cells. In the present study we explored the cell cycle regulation of FPGS expression in ALL cell models. The mRNA and protein levels were found to be higher in G1 in preparation for DNA synthesis during S-phase. Our data

indicates that additional members of the multiprotein complex described above, such as Rb and E2F, participate in the cell-cycle dependence of FPGS expression. It has been reported that cell cycle progression, DNA replication, mitosis, and proliferation are regulated by the balance of E2F activities [38,39]. Within folate pathway genes, DHFR and TYMS expression were found to be controlled by the E2F transcription factor [37,36]. More specifically, expression of DHFR during G1 to S-phase progression is regulated by the Rb/E2F pathway and the Sp1 transcription factor, both interacting with HDAC1 [36]. The Rb protein is known to repress gene transcription by directly binding to the transactivation domain of E2F and by binding to the promoter of these genes as a complex with E2F that recruits HDAC to E2F target promoters to close the chromatin conformation and repress transcription. E2F binding sites have been detected in 7 folate pathway related genes (DHFR, TYMS, MTHFD1, MTHFD2, ATIC, GART, and RUVBL2) [13]. Herein, we report the identification of a putative E2F binding site (GTTTGGGGCGG) at position -97 within the FPGS promoter region [42]. In addition, we demonstrate that similar to NFY, both E2F and Rb can physically bind to the native chromatin structure of the FPGS promoter region during cell cycle progression. Therefore, our data strongly suggest that NFY, Sp1, Rb and E2F transcription factors interact together and are responsible for the cell cycle-dependence of FPGS expression.

The prognostic significance of non-random translocations in ALL results from the molecular phenotypes that influence cell survival and response to chemotherapy. These phenotypes determine distinct patterns of expression of folate-related genes described by us and others [13], which influence the intracellular accumulation of MTX-PG and sensitivity to classical antifolates. In this study, we demonstrate that selected ALL fusion proteins bind to the promoter region of FPGS and propose a hypothetical model for decreased FPGS mRNA expression in cells expressing TEL-AML1 and possibly other fusions (Figure 7). As part of a multiprotein complex, NFY and Sp1 recruit HDAC1 to the FPGS promoter region to regulate its expression. In cells expressing TEL-AML1, HDAC1 interacts with TEL-AML1 to down-regulate FPGS mRNA expression. TEL is a member of the ETS family of transcription factors, and it is known to interact with several co-repressors including mSin3A and N-CoR, a component of the nuclear receptor co-repressor that complexes with HDACs [43,28,41]. Similar models depicting the molecular mechanisms of transcriptional repression by TEL-AML1 in ALL cells have been reported by others [30,33,44,45,46]. All these models support the notion that the TEL-AML1 fusion recruits co-repressors and HDAC to trans-repress AML1 target genes in ALL [32,45,31]. Consequently, the proposed model of epigenetic regulation of FPGS expression by TEL-AML1 is supported by the experimental data presented herein and is consistent with a large body of reported literature.

In conclusion, this study is the first to demonstrate the causal relationship between the presence of E2A-PBX1 and TEL-AML1 gene fusions and decreased FPGS mRNA expression, which will subsequently lead to lower intracellular accumulation of long-chain MTX-PGs. Our data suggest that chromatin remodeling is responsible for altered FPGS mRNA expression in cells expressing E2A-PBX1 and TEL-AML1. We present evidence that TEL-AML1, mSin3A, and HDAC1 interact with the chromatin structure of the FPGS promoter region to down regulate its expression. In addition, we found that FPGS expression is up-regulated by this multiprotein complex in G1-phase prior to progress into S-phase in anticipation of increased folate and nucleoside requirements for DNA replication. Our findings demonstrate that FPGS expression in lymphoid cells is regulated by chromatin remodeling through interactions between NFY, Sp1, E2F, Rb, and HDAC factors binding to the promoter region of the FPGS gene.

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Figure 1.

Level of MTX polyglutamation genes (FPGR and GGH), MTX transporters (ABCC1, ABCG2, and SLC19A1) and MTX target genes (DHFR and TS) mRNA expression in Bp-ALL primary cells with chromosomal abnormalities and non-random translocations present in ALL subtypes. Levels of folate-related gene mRNA were determined using real-time qRT-PCR as described in Materials and Methods. Box plots with medians, quartiles, and ranges of $\log$ mRNA expression are depicted. Data were from 109 Bp-ALL primary samples with t(1,19)/E2A-PBX1 (n=29), t(12,21)/TEL-AML1 (n=26), trisomies 4/10 (n=28), or hyperdiploidy (26) phenotypes. P values were determined using Kruskal-Wallis and Global F-tests.

Figure 2.

Level of FPGS mRNA expression in NALM6 stable cell lines expressing TEL-AML1 or E2A-PBX1. NALM6 stable cell lines were transfected by nucleofection with a pCI-Neomycin vector expressing E2A-PBX1 or TEL-AML1, and transfectants were selected with Geneticin (600 $\mu\text{g/ml}$). Expression of both fusions was confirmed by qRT-PCR (A) and Western blotting (B). Level of FPGS mRNA expression was determined using real-time qRT-PCR (C). Data were normalized to level of FPGS expression detected in NALM6 cells. Experiments were performed at least two times in quadruplicate.

Figure 3.

FPGS mRNA expression and promoter activity in cells expressing TEL-AML1 or E2A-PBX1 fusion proteins. A) Relative level of FPGS mRNA expression in cell line models REH (TEL-AML1) and RCH-ACV (E2A-PBX1) determined by qRT-PCR. Data were normalized to level of FPGS mRNA expression detected in NALM6 cells. B) FPGS null mutant AUXB1 cells were triple transfected with constructs expressing FPGS-luciferase, CMV- β gal, and either E2A-PBX1, TEL-AML1, or empty vector pCDNA6/V5-His/C using nucleofection as described in Materials and Methods. In the transient transfected cells, expression of TEL-AML1 and E2A-PBX1 mRNA and protein was confirmed by qRT-PCR and Western blots. Levels of luciferase and β -galactosidase activities were assayed using the dual-light reporter gene assay system. Experiments were performed at least two times in quadruplicate. Bars represent mean $\pm$ SD.

Figure 4.

Chromatin immunoprecipitation of AML1, HDAC1, and mSin3A proteins in REH cells. Chromatin extracted from REH cells was immunoprecipitated with AML1, HDAC1, and mSin3A antibodies and purified DNA were subjected to PCR using primer sets designed to amplify a 449 bp fragment of the FPGS promoter region as described in Materials and Methods. All pre-cleared chromatin supernatant samples yielded similar amounts of the 449 bp PCR amplified DNA fragment from the FPGS promoter region. The ethidium bromide-stained agarose gel shows a representative sample of input DNA (5%) from pre-cleared chromatin supernatants, PCR products from immunoprecipitated chromatin using specific antibodies, normal rabbit IgG, no antibody, and PCR negative control. ChIP assays for AML1, HDAC1, and mSin3A were performed in duplicate.

Figure 5.

Level of FPGS expression during cell cycle progression. NALM6 cells were arrested in S-phase using hydroxyurea and cultured in drug-free medium to release synchronized cells in S and G1 phases. A) Relative level of FPGS mRNA and protein expression during cell cycle as determined by real-time qRT-PCR and Western blots. B) Level of FPGS protein expression in NALM6 cells arrested in G1 and S phases. C) ChIP of acetyl histone H3 protein in NALM6 cells treated with HDACi (SAHA) as described in Materials and Methods. The top ethidium bromide (EtBr)-stained agarose gel shows PCR products from immunoprecipitated chromatin using specific antibodies, normal rabbit IgG, and no antibody. The bottom EtBr-stained agarose gel shows input DNA (5 %) from pre-cleared chromatin supernatants of all ChIP DNA samples. D) Level of acetyl histone H3 protein expression in NALM6 cells arrested in G1 and S phases as determined by Western blot. Samples represent extracts obtained from three independent experiments.

Figure 6.

Chromatin immunoprecipitation of E2F, HDAC1, NFYB, and Rb proteins in NALM6 cells. Chromatin extracted from NALM6 cells was immunoprecipitated with E2F, HDAC1, NFYB, and Rb antibodies and DNA subjected to PCR amplification of the 449 bp fragment from the FPGS promoter region as described in Materials and Methods. The top ethidium bromide stained agarose gel shows PCR products from ChIP samples and PCR negative control. The bottom stained agarose gel shows input DNA (10%) from pre-cleared chromatin supernatants from all ChIP DNA samples.

Figure 7.

Proposed model for decreased FPGS mRNA expression in cell expressing TEL-AML1. A) As part of a multiprotein complex, NFY and Sp1 recruit HDAC1 to the FPGS promoter region to regulate its expression. B) In cells expressing TEL-AML1, HDAC1 interacts with TEL-AML1 and possibly other regulatory factors (e.g., NCoR, mSin3A, Rb, etc.) to down regulate FPGS mRNA expression. C) Treatment with an HDAC1 inhibitor (e.g. SAHA or NaBu) will inhibit HDAC1 activity and disrupt interactions between Sp1, NFY and HDAC1 leading to FPGS promoter activation.