The intricate role of Smads in the regulation of FPGS gene expression

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Antifolates are important components of treatment regimens of hematological malignancies and solid tumors. Following cellular uptake, antifolates undergo polyglutamylation, catalyzed by the enzyme folylpoly-γ-glutamate synthetase (FPGS), which is crucial for their intracellular retention and cytotoxic activity. Hence, loss of FPGS activity results in diminished antifolate polyglutamylation and antifolate resistance. Aberrant TGF-β/Smad signaling is thought to be a major contributor to leukemogenesis, as members of this pathway are negative regulators of hematopoiesis. Recently we characterized the human antifolate resistant leukemia cell line MTA C-3 which lost 97% of its cellular FPGS activity and harbors a heterozygous point mutation in exon12 of FPGS. This resulted in the loss of 99% of the expression of the wild type FPGS allele, while the expression of the mutant allele was retained. Research into the molecular mechanism underlying the selective silencing of the wild type allele in these cells established exon12 of FPGS as an intragenic transcriptional regulator, with the ability to drive transcription in vitro. We further showed that exon12 can be occupied by many transcription factors and chromatin remodeling proteins (e.g. Smad4/Ets-1, HP-1 and Brg1) in vivo and that the expression of FPGS is inversely correlated with the binding of a Smad4/Ets-1 complex to exon12, both in acute lymphoblastic leukemia cells and acute myeloid leukemia blast specimens. Here we further demonstrate the possible roles of Smads in the regulation of FPGS gene expression. We found that treatment of MTA C-3 cells with 5-Aza-deoxycytidine and trichostatin A results in repression of the mutant allele as well as in decreased expression of FPGS in drug sensitive parental CCRF-CEM cells. This repression correlated with an increased expression of Smad3 and the inhibitory Smad7. These findings bear important implications for the rational overcoming of antifolate resistance in leukemia.

Keywords: Antifolate resistance; Leukemia; FPGS, Intragenic regulatory element; Smad transcription factors


Introduction

Folates are essential vitamins that serve as cofactors in various metabolic processes including DNA methylation and biosynthesis of nucleotides and amino acids [1, 2]. Antifolates are folate antagonists which block crucial enzymes in the folate metabolic pathway, thereby disrupting DNA replication and inducing apoptosis in rapidly dividing cells [1]. The enzyme folylpoly-γ-glutamate synthetase (FPGS) catalyzes the addition of 1-9 glutamate residues to folates and polyglutamatable antifolates, which are then retained inside the cells due to markedly reduced affinity of (anti)folate exporters to polyglutamated (anti)folates [3, 4]. While the affinity of the drug efflux transporters (e.g. ABC1 and ABCG2) to polyglutamated antifolates dramatically decreases, the affinity of the target enzymes (i.e.
DHFR, TS, GARTF) increases by ~100-fold [5]. Therefore, FPGS plays a key role in the cytotoxic activity of antifolates and in the treatment outcome of patients suffering from various malignancies. The pharmacological efficacy of antifolate-containing chemotherapy was shown to be correlated with the cellular accumulation of antifolate polyglutamates in cancer patients with acute lymphoblastic leukemia (ALL) and osteosarcoma [6-9]. Although antifolates are key components of treatment regimens of ALL, blasts retrieved from acute myeloid leukemia (AML) patients do not accumulate long-chain antifolate polyglutamates [10], and AML patients are therefore considered resistant to these important drugs.

We recently discovered that the antifolate resistant human leukemia cell line MTA C-3 harbors a heterozygous missense mutation in exon12 of FPGS (i.e. R363Q substitution) that is predicted to abolish ATP-binding by FPGS, thereby leading to a 97% loss of FPGS activity. While the allele that harbors this inactivating mutation was fully expressed, the wild type (WT) allele was completely silenced [11]. This silencing was correlated with the differential binding of a complex comprised of the factors Sma- and Mad-related 4 (Smad4) and avian erythroblastosis virus E26 (v-ets) oncogene homolog-1 (Ets-1) to exon12 of the WT allele and consequent recruitment of epigenetic modifying proteins. We identified Smads as modulators of FPGS gene expression and exon12 of FPGS as an important regulatory element by demonstrating that the expression of FPGS in blasts from AML patients is inversely correlated with the binding of the Smad4/Ets-1 complex to its own exon12 [11].

The Smad transcription factors (TFs) are the intracellular effectors of TGF-β signaling. While Smad4 is constitutively expressed, it translocates to the nucleus only when in complex with a phosphorylated Smad. The latter is activated by TGF-β (Smad2 and Smad3) or in response to bone morphogenetic proteins (Smad1, Smad5 and Smad8) [12]. TGF-β is considered to be an effective negative regulator of hematopoiesis, primarily in the myeloid cell lineage, since it induces cell cycle arrest in committed progenitors by down-regulating cyclins, cyclin-dependent kinases and c-myc [13].

Here we provide further evidence for the possible roles of Smad proteins in the regulation of FPGS expression. We demonstrate that the expression of the mutant allele in MTA C-3 cells and the expression of FPGS in parental drug sensitive CCRF-CEM cells are reduced upon exposure to 5-Aza-deoxycytidine (5-Aza-CdR) and trichostatin A (TSA). This is correlated with an increase in the expression of Smad3, previously shown to bind specific repressive elements [14, 15], and Smad7, an inhibitory Smad which represses Smad signaling [16, 17].

Materials and Methods

Cell culture

The human leukemia cell line CCRF-CEM and its antifolate-resistant MTA C-3 subline were maintained as previously described [11].

5-Aza-CdR and TSA treatment

Cells were seeded at a concentration of 2X10^5 cells/ml and 1µM 5-Aza-CdR (Sigma-Aldrich) was added to the growth medium. Following 32h of exposure to 5-Aza-CdR, TSA (Sigma-Aldrich) was added to a final concentration of 0.1µg/ml for the remaining 16h. RNA was extracted from the cells using the TRI-reagent kit (Sigma-Aldrich) and reverse transcription was carried out using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems-Life Technologies) according to the manufacturer’s instructions.

Real-time PCR analysis

Allele-specific real-time PCR was performed as previously described [11]. Real-time PCR analysis of gene expression was performed as previously described [11]. The primers used to quantify the expression of Smad2-4 and Smad7 are depicted in Table 1.

Methylation-sensitive restriction enzyme PCR (MSRE-PCR)

Genomic DNA (gDNA) was extracted from MTA C-3 cells following 5-Aza-CdR and TSA treatment using the DNeasy Blood & Tissue Kit (QIAGEN). The gDNA was digested with the methylation-sensitive enzymes DraIII and FspI (NEB) and the methylation insensitive enzyme Stul (NEB), which was used as a control. 250ng of gDNA was digested using 2.5U of each enzyme and the reaction was performed according to the manufacturer’s instructions. Exon12 of FPGS, as well as exon2 and the minimal promoter were PCR-amplified from 12.5ng of digested gDNA and PCR products were resolved on 1% agarose gels containing ethidium bromide. The primers used for this assay are depicted in Table 1.

Bioinformatics analysis

MatInspector (Genomatix) was used to analyze the MP and exon12 of the human FPGS for putative TF binding sites. Putative CpG island predictions were performed by Methyl primer express (Applied Biosystems-Life Technologies) using the parameters: 300-2,000bp CpG island
length, over 50% GC content and CpG observed/CpG expected ratio >0.6.

Results

We previously found that various elements modulate the expression of the WT and mutant FPGS alleles in antifolate-resistant MTA C-3 leukemia cells (Figure 1) [11]. Since epigenetic mechanisms govern the expression of the WT FPGS allele (i.e. high nucleosome density and histone H3K9 dimethylation), we explored the impact of the epigenetic modifiers 5-Aza-CdR and TSA, which decrease DNA/histone methylation [18, 19] and histone deacetylation [20], respectively, on the expression of FPGS. Hence, we treated MTA C-3 cells with TSA (0.1μg/ml, 16h) and/or 5-Aza-CdR (1μM, 48h) and determined the expression of each allele by allele-specific real-time PCR. To our great surprise, the expression of the WT FPGS allele was not altered by this treatment, whereas that of the mutant allele was reduced by 40-60% (Figure 2A). We recently showed that the genomic region of exon12 of FPGS is completely methylated in both healthy and leukemic cells [11]. Therefore, we first assessed whether or not the DNA methylation status of exon12 was altered following treatment with 5-Aza-CdR and TSA using the MSRE-PCR assay [21]. The methylation-sensitive restriction enzymes DraIII and FspI have restriction sites within exon12 of FPGS and were therefore used to digest gDNA isolated from 5-Aza-CdR- and TSA-treated cells. Following digestion, a PCR reaction was performed using primers which amplify the genomic region of exon12. We found that both enzymes were not able to cut exon12 of FPGS (Figure 3), thereby indicating that this region remains methylated in MTA C-3 cells following 5-Aza-CdR and TSA treatment (digestion of gDNA from parental CCRF-CEM cells produced the same results, data not shown). To verify that the gDNA was indeed fully digested with the restriction enzymes, we amplified a segment from the promoter and exon2 of FPGS, which harbor restriction sites for DraIII and FspI, respectively, and were previously found to be unmethylated using bisulfite-sequencing assay [11] (Figure 3).

Our recent work suggested that the expression of the mutant allele is enhanced by the binding of a Smad2/Smad4 complex to a newly formed Smad binding element (SBE) in the mutant exon12 sequence of MTA C-3 cells [11]. We therefore determined the possible alterations in the expression of Smads following exposure to 5-Aza-CdR and TSA. The expression of Smad2 and Smad4 remained unchanged (data not shown). In contrast, the expression of Smad3 as well as Smad7, both of which are completely silenced in MTA C-3 cells, was markedly increased by up to 8-fold after exposure to 5-Aza-CdR and TSA (Figure 2B). Indeed, bioinformatics analysis predicts the existence of a long and dense CpG island in the promoter of both Smad3 and Smad7, thus suggesting a possible explanation for the restoration of their gene expression following 5-Aza-CdR and TSA. Smad7 is an inhibitory trans-acting factor which was shown to bind to SBEs, thereby interfering with the binding of active Smad complexes [16, 17]. As such, it should affect the expression of the mutant allele in MTA C-3 cells, which harbors an SBE, but not the WT FPGS allele. To confirm that exposure to 5-Aza-CdR and TSA represses the expression of only the mutant allele, and since the WT allele is already completely repressed, we treated both MTA C-3 and their parental CCRF-CEM cells with these epigenetic modifiers. Real-time PCR analysis of the expression of FPGS, using primers residing in the second exon of FPGS, in both CCRF-CEM and MTA C-3 cells after exposure to 5-Aza-CdR and TSA surprisingly revealed that the expression of FPGS was reduced by ~40% in both leukemia cell lines (Figure 2C).

| Table 1. Primers used in various applications |
| Smad2 | Fw | AAGAGGAGTGCGCTTATACTAC |
| | Rv | ACCCTTCACAACACTTAC |
| Smad3 | Fw | CACTCCCCGCCGCTGGAAG |
| | Rv | ATGGCCCCGTAGTCTGG |
| Smad4 | Fw | CTGCTCCCTGATTTTGTTCC |
| | Rv | CAGGCCAGCTTCTGTCTA |
| Smad7 | Fw | CGGTGTCAGAGAAGCTAGAGG |
| | Rv | CAGGTATCTGGATAAGG |
| FPGS Int11-ex13 | Fw | GTCATCGCTTCTTGCTCCCC |
| | Rv | CAGTCCCCGGTACATTGA |
| FPGS promoter | Fw | GTCTCTGCTTATCCCCAGCC |
| | Rv | GTCTCGAATTCCCCAGCC |
| FPGS ex2-ex3 | Fw | CGCATGCTCAATACCTTG |
| | Rv | TTCCCCCTCGTCCAGTAC |
Our recent study showed no binding of Smad2/4 to either exon12 or the minimal promoter (MP) of FPGS in parental CCRF-CEM cells, therefore the reduction in the expression of FPGS in these cells cannot be due to the inhibitory effect of Smad7 on Smad-binding. However, Smad3 was previously shown to bind to a specific repressive SBE (RSBE) with a sequence distinct from the canonical SBE, thereby repressing the expression of c-myc [14] and lysophosphatidic acid receptor 1 [15]. We screened the promoter and the entire genomic sequence of FPGS for this RSBE and found one such element (i.e. TTGGCGGG) in the MP of FPGS. To determine whether or not binding of Smad3 may contribute to the decreased expression of FPGS after 5-Aza-CdR and TSA treatment in CCRF-CEM cells, we determined the expression level of Smad3 after exposure of the cells to these agents and found it to be induced by 3-5 fold (Figure 2D).

Discussion

We recently found that the antifolate resistant human leukemia cell line, MTA C-3, harbors a heterozygous mutation in exon12 of FPGS, leading to an R363Q substitution. Strikingly, differential allelic expression (DAE), based on the complete silencing of the WT allele of FPGS, resulted in the exclusive expression of the mutant allele which encodes an inactive FPGS enzyme, thus leading to antifolate drug resistance in MTA C-3 cells.

While DAE in mammals was described in several key biological processes such as genomic imprinting, X chromosome inactivation and the production of interleukins and antibodies (reviewed by Cedar, H. and Bergman, Y. [22]), allele-specific silencing has not been implicated in drug resistance phenomena. Several mechanisms were shown to underlie DAE: 1) DNA methylation controls the predetermination of imprinted genes in the gametes [23], 2) Non-coding RNAs direct the inactivation of the X chromosome in females [24], 3) Allele-specific epigenetic silencing modifications (i.e. H3K9me2 and HP-1 binding) determine random allelic exclusion in immune-cell development [22]. DAE is also an emerging contributing factor to human disease; for example, allele-specific chromatin remodeling in the ZPBP2/GSDMB/ORMDL3
locus was shown to be associated with increased risk of asthma and autoimmune disease \cite{25}. Moreover, we have previously identified DAE in the human multidrug efflux transporter ABCG2 harboring a frequent A421 heterozygous polymorphism as a novel mechanism of multidrug resistance \cite{26}. In that case, allele-specific gene amplification of the WT C421 allele rendered human non-small cell lung cancer cells >30-fold more resistant to ABCG2 substrate-drugs than their drug sensitive parental counterparts.

The DAE in MTA C-3 cells is mediated by the binding of an assortment of factors to exon12 of \textit{FPGS}, the latter of which was found to have transcriptional regulatory capabilities \cite{11}. An inverse correlation was observed between the binding of the TFs Smad4 and Ets-1 to exon12 of \textit{FPGS} and \textit{FPGS} gene expression. Specifically, we have shown that Smad4 and Ets-1 bind to the WT but not the mutant allele of \textit{FPGS} in MTA C-3 cells, thereby leading to recruitment of epigenetic modifying factors and consequent selective silencing of the WT allele. Moreover, in blasts from AML patients with decreased expression of \textit{FPGS} this Smad4/Ets-1 complex was found to bind exon12 of \textit{FPGS}. Thus, we demonstrated that TFs occupy exon12 \textit{in vivo} and may lead to decreased \textit{FPGS} gene expression.

Expanding the scope of these findings, we here show that exposure of MTA C-3 cells to 5-Aza-CdR and TSA results in reduced expression of the mutant \textit{FPGS} allele. While no changes were found in the DNA methylation status of exon12, we did observe restoration of gene expression of
Smad3 and Smad7, both of which harbor a putative CpG island in their promoter. These findings are in agreement with a recent publication demonstrating that the expression of Smad7, which is suppressed by TGF-β in hepatic stellate cells, was induced by silencing of DNA methyltransferase 1 as well as exposure of the cells to 5-Aza-CdR [27]. Since Smad7 binds to SBEs with high affinity, thus interfering with the binding of activated Smad complexes [16, 17], Smad7 can bind to the SBE in the mutant exon12 in MTA C-3 cells following 5-Aza-CdR and TSA treatment, thereby impeding Smad2/Smad4 binding to this element. This would suggest that the Smad2/Smad4 complex has a positive role in the regulation of the expression of FPGS. However, treatment with 5-Aza-CdR and TSA reduced the expression levels of FPGS by 40% in both MTA C-3 and CCRF-CEM cells, whereas Smad2/Smad4 were not found to occupy exon12 or the MP of FPGS in CCRF-CEM cells [11]. Hence, the altered expression of FPGS following exposure to 5-Aza-CdR and TSA must be influenced by other trans-acting factors, rather than by Smad7. We found that the expression of the silenced Smad3 was also induced in both MTA C-3 and CCRF-CEM cells after exposure to 5-Aza-CdR and TSA. Smad3 was shown to bind to a specific repressive sequence termed RSBE, which overlaps a consensus E2F1 binding site, and inhibit the expression of c-myc [14] and lysophosphatidic acid receptor 1 [15]. Analysis of the promoter and the entire FPGS gene revealed the existence of the same RSBE-E2F site at the MP of FPGS, which can be bound by Smad3 and thereby repress gene expression following treatment with 5-Aza-CdR and TSA. It appears that Smad family members have an intricate regulatory role in modulating FPGS expression, both as activators and as suppressors. Further research is warranted regarding the role that Smads play in the regulation of other folate metabolic genes.

Marked down-regulation of FPGS leads to a decrease in the cellular folate pools [28] which in turn results in decreased nucleotide pools. The latter facilitates rapid cell-cycle arrest as we have previously shown in response to hypoxia, which induced a simultaneous reduction in the expression of folate metabolism pathway enzymes and transporters [29]. Exon12 of FPGS also harbors nuclear respiratory factor (NRF)-1 binding sites and a hypoxia response element that may contribute to the hypoxia-induced silencing of FPGS. Moreover, bioinformatics analysis of exon12 and the MP of FPGS revealed that FPGS is potentially regulated by c-myc, as several E-box elements are present at both regulatory elements. Interestingly, TGF-β, which is known to be a negative regulator of hematopoiesis, induces cell cycle arrest by down-regulation of c-myc mediated by the binding of Smad3 to RSBE [14]. Thus, up regulation of Smad3 after 5-Aza-CdR and TSA treatment may repress the expression of FPGS by directly binding to the MP of FPGS and by down-regulating c-myc. Clearly, a more detailed analysis of these sites is warranted in order to achieve a comprehensive understanding of the role of exon12 in the expression of FPGS.

Conflicting interests

The authors have declared that no competing interests exist.

References

7. Whitehead VM, Rosenblatt DS, Vuchich MJ, Shuster JJ, Witte A


14. Frederick JP, Liberati NT, Waddell DS, Shi Y and Wang XF. Transforming growth factor beta-mediated transcriptional repression of e-myc is dependent on direct binding of Smad3 to a novel repressive Smad binding element. Mol Cell Biol 2004; 24:2546-2559.


