Supplemental Information for:

Folate Pathway Gene Expression Differs in Subtypes of Acute Lymphoblastic Leukemia and Influences Methotrexate Pharmacodynamics

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Figure S 1: The methotrexate polyglutamate (MTXPG) pathway.

Schematic diagram depicting proteins which are known to contribute to cellular methotrexate polyglutamate (MTXPG) accumulation. MTX enters cells primarily via the reduced folate carrier (RFC or SLC19A1). Other import routes include folate receptors (FRs), and in acidic milieu 'low pH MTX transport activity' (low pH). In the cytosol, MTX is glutamylated to MTXPG2-7 by folylpolyglutamate synthase (FPGS). In lysosomes, MTXPGs can be converted back to the parental drug by gamma-glutamyl hydrolase (GGH). MTX can be effluxed by ABC-transporters, namely multidrug resistance-associated proteins 1 to 4 (MRP1-4 or ABCC1-4), breast cancer resistance protein (BCRP or ABCG2), and under certain conditions by P-glycoprotein (MDR1 or ABCB1). In addition, short chain MTXPGs are substrates for BCRP. Unidirectional importers are in white, unidirectional exporter in black, and bidirectional transporter in grey.

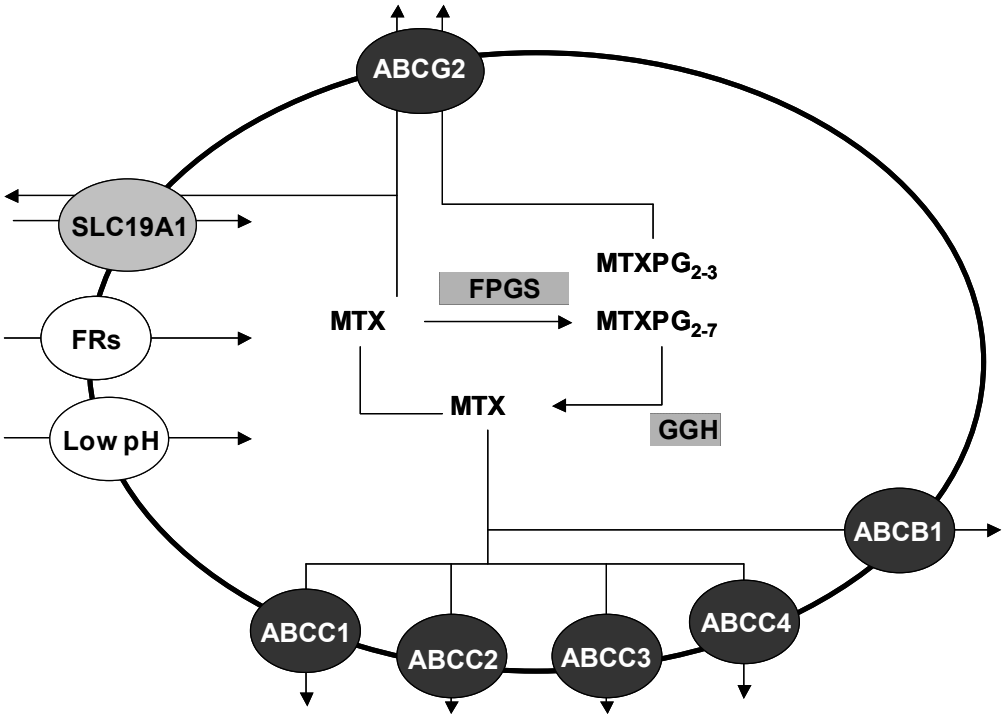


Table S 1: 'Candidate' folate pathway genes

Selected 32 'candidate' genes (53 probe sets) involved in cellular folate/antifolate metabolism. Genes were selected from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database for 'Human' and **A.** 'Folate biosynthesis', **B.** 'One carbon pool by folate'. MTX transporter and additional genes known to be of importance in folate/antifolate metabolism but not found in KEGG pathway database are listed in **C.** Probes (and corresponding genes) that did not pass the 'detection call' filter (>95% absent calls) and were therefore not included into further analyses are highlighted in grey (9 genes). Superscripted numbers ($^{1\text{-}4}$) indicate highly correlated transcripts (correlation coefficient r >0.4) within a set of probes encoding for one gene. These transcripts were combined into one expression variable as described in the Methods and Results of the main manuscript. Probe sets representing the same gene with expression not correlated, were considered independent transcripts (i.e., for *GART* and *PPAT*).

A. 'Folate biosynthesis'

Gene Symbol	Probe Set ID	Public ID	Gene name	Chromosome
RUVBL2	201459_at	NM_006666	RuvB, E. coli, homolog-like 2	19q13.3
FPGS	202945_at	NM_004957	folylpolyglutamate synthase	9cen-q34
SPR	203458_at	AI951454	sepiapterin reductase	2p14-p12
GGH	203560_at	NM_003878	gamma-glutamyl hydrolase	8q12.2
GCH1	204224_s_at	NM_000161	GTP cyclohydrolase 1	14q22.1-q22.2
PTS	209694_at	M97655	6-pyruvoyltetrahydropterin synthase	11q22.3-q23.3
ALPI	211618_s_at	M31008	alkaline phosphatase, intestinal	2q37.1
MTHFD1	202309_at	NM_005956	methylenetetrahydrofolate dehydrogenase 1	14q24
DHFR	202532_s_at[1], 202533_s_at[1], 202534_x_at[1], 48808_at[1]	BC000192, BC003584, NM_000791, AI144299	dihydrofolate reductase	5q11.2-q13.2
TYMS	202589_at, 217684_at, 217690_at	NM_001071, BG281679, BG281679	thymidylate synthetase	18p11.32

B. 'One carbon pool by folate'.

Gene Symbol	Probe Set ID	Public ID	Gene product	Chromosome
MTHFD2	201761_at	NM_006636	methylenetetrahydrofolate dehydrogenase 2	2p13.1
MTHFS	203433_at	NM_006441	5,10-methenyltetrahydrofolate synthetase	15q24.3
ATIC	208758_at	D89976	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	2q35
GART	210005_at, 212378_at[2], 212379_at[2], 216990_at, 217445_s_at[2]	D32051, NM_000819, BE966876, AF008655, AF008655	phosphoribosylglycinamide formyltransferase,	21q22.1
FTCD	220604_x_at	NM_006657	formiminotransferase cyclodeaminase	21q22.3
SHMT1	209980_s_at	L23928	serine hydroxymethyltransferase 1	17p11.2
MTR	203774_at	NM_000254	5-methyltetrahydrofolate-homocysteine methyltransferase	1q43
AMT	204294_at	NM_000481	aminomethyltransferase	3p21.2-p21.1
FTHFD	205208_at, 215798_at	NM_012190, AL133015	formyltetrahydrofolate dehydrogenase	3q21.2

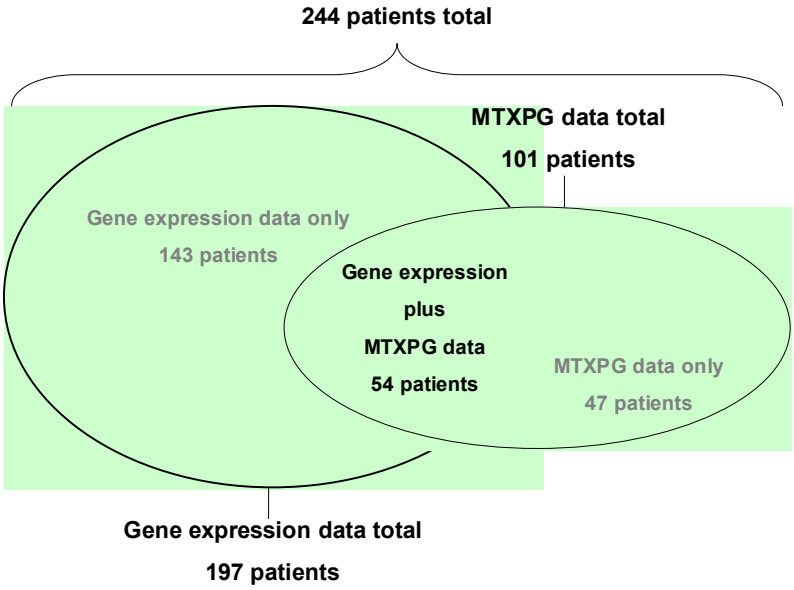
C. Other genes involved in folate/antifolate metabolism

Gene Symbol	Probe Set ID	Representative Public ID	Gene product	Chromosome
MTHFR	206800_at, 217070_at, 217071_s_at	NM_005957, AJ249275, AJ249275	5,10-methylenetetrahydrofolate reductase (NADPH)	1p36.3
PPAT	209433_s_at, 209434_s_at	AI457120, U00238	phosphoribosyl pyrophosphate amidotransferase	4q12
FTH1	200748_s_at[3], 214211_at[3]	NM_002032	ferritin, heavy polypeptide 1	11q13
ABCC1	202804_at[4], 202805_s_at[4]	AI539710, NM_004996	ATP-binding cassette, sub-family C (CFTR/MRP), member 1, MRP1	16p13.1
ABCC4	203196_at	AI948503	ATP-binding cassette, sub-family C (CFTR/MRP), member 4, MRP4	13q32
FOLR1	204437_s_at	NM_016725	folate receptor 1 (adult)	11q13.3-q14.1
FOLR2	204829_s_at	NM_000803	folate receptor 2 (fetal)	11q13.3-q13.5
ABCC2	206155_at	NM_000392	ATP-binding cassette, sub-family C (CFTR/MRP), member 2, MRP2	10q24
FOLR3	206371_at	NM_000804	folate receptor 3 (gamma)	11q13
ABCC3	208161_s_at, 214979_at, 209641_s_at	NM_020037 AK000791, AF009670	ATP-binding cassette, sub-family C (CFTR/MRP), member 3, MRP3	17q22
ABCG2	209735_at	AF098951	ATP-binding cassette, sub-family G (WHITE), member 2, BCRP	4q22
SLC19A1	209775_x_at, 209776_s_at, 209777_s_at, 211576_s_at	AI917627, U15939, AF004354, BC003068	solute carrier family 19 (folate transporter), member 1, reduced folate carrier	21q22.3
ABCB1	209993_at, 209994_s_at	AF016535, AF016535	ATP-binding cassette, sub-family B (MDR/TAP), member 1, Pgp	2q24

2. Patients

Figure S 2: Summary of patients included in the study.

Only patients with unequivocally proven ALL subtypes were included, that is a result (positive or negative) for *BCR-ABL, TEL-AML1, E2A-PBX1* and *MLL* rearranged was required, if any one of these tests were not done or uncertain the patient was excluded.



3. Real time RT-PCR

Gene expression was also determined by real time RT-PCR assays (reverse transcription polymerase chain reaction) in four patient samples for four selected genes, i.e., breast cancer resistance protein, *ABCG2*; 5-aminoimidazole-carboxamid ribonucleotide formyltransferase, *ATIC*, thymidylate synthetase, *TYMS*, and methylenetetrahydrofolate dehydrogenase 1, *MTHFD1*. Reactions were performed using TaqMan Universal PCR Master Mix Kit and the 7900-sequence-detection system (Applied Biosystems).

Two-hundred ng of total RNA was treated with Dnase I and reverse transcribed using Superscript II RNase H^- reverse transcriptase and oligo dT primers (Invitrogen). Additionally, controls that contained either no template or no reverse transcriptase were included as negative controls in each run. Aliquots (1 µl) of RT reaction mixture (20 µl) were used for quantification of *TYMS, MTHFD1, ABCG2, ATIC* and *Rnase P* gene expression. The housekeeping gene, *Rnase P* (Applied Biosystems), was used for normalization.

The total volume of PCR reaction was 50 µl of RT-product, 400 nM each of the forward and reverse primers, 250 nM of probe, and 1X master mix. The following thermal cycling parameters were used: two minutes at 50^OC (activation of UNG enzyme to remove the carry-over PCR products), ten minutes at 95^OC to activate AmpliTaq Gold DNA polymerase, 15 seconds at 95^OC to denature and one minute at 60^OC for annealing and extension, for a total of 45 cycles.

To estimate the amount of each of the four mRNAs in the four patient samples, we used linear regression analysis based on a standard curve representing six serial dilutions of cDNA made from the Nalm6 human leukemia cells (American Type Culture Collection).

Real-time RT-PCR standard curves showed r^2>0.99 for *TYMS, MTHFD1, ABCG2, ATIC* and *Rnase P* (data not shown). All unknown samples were analyzed in duplicate in parallel with a standardization series from Nalm6 cDNA. The relative quantification values of genes for each sample were calculated based on their C_T (threshold cycle number) value and the corresponding standard curves.

The correlation (r^2) between mRNA expression determined by real-time RT-PCR and microarray analyses was 84% for *ATIC*, 91% for *TYMS*, 75% for *ABCG2*, and 88% for *MTHFD1*, thereby confirming expression levels determined by the gene expression array.

Figure S 3: Real-time (TaqMan) RT-PCR vs. GeneChip

Real-time RT-PCR results are plotted versus expression levels determined by GeneChip results for *ATIC, TYMS, ABCG2* and *MTHFD1* in four patients.

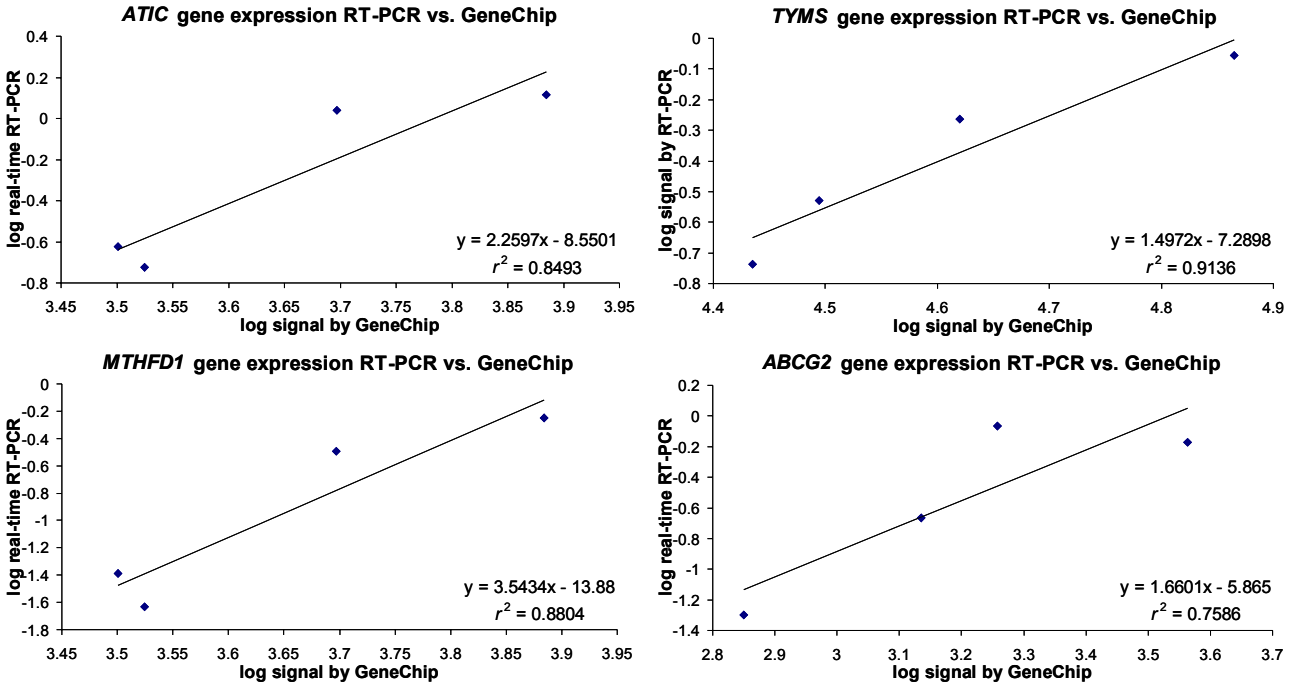
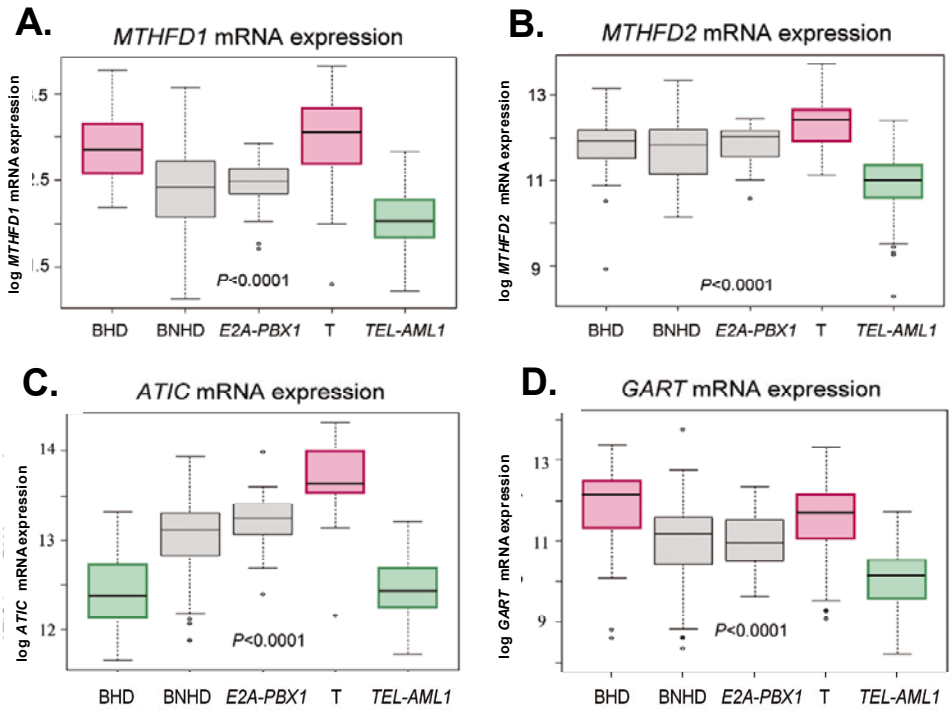


Figure S 4: Gene expression pattern of four folate pathway genes discriminating ALL genetic subtypes.

Box plot with medians, quartiles and range without outliers of log mRNA expression (HG-U133A GeneChip) are depicted for **A.** Methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*), **B.** Methylenetetrahydrofolate dehydrogenase 2 (*MTHFD2*), **C.** 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (*ATIC*), and **D.** Phosphoribosylglycinamide formyltransferase (*GART*). Data from 197 patients were plotted (BHD, *n*=42; BNHD, *n*=58; *E2A-PBX1*, *n*=21; T-ALL, *n*=35; and *TEL-AML1*, *n*=41; Kruskal Wallis test).



4. Correlation between MTXPG accumulation and expression of folate pathway genes in ALL

Figure S 5: Total MTXPG levels versus *TYMS* and *MTHFD1* mRNA expression.

Data are shown for ALL cells from 54 patients. P-values are for Spearman correlation. BHD subtypes (*n*=9) are depicted in red, BNHD (*n*=22) in blue, *E2A-PBX1* (*n*=3) in yellow, T-ALL (*n*=9) in black, and *TEL-AML1* (*n*=11) in green.

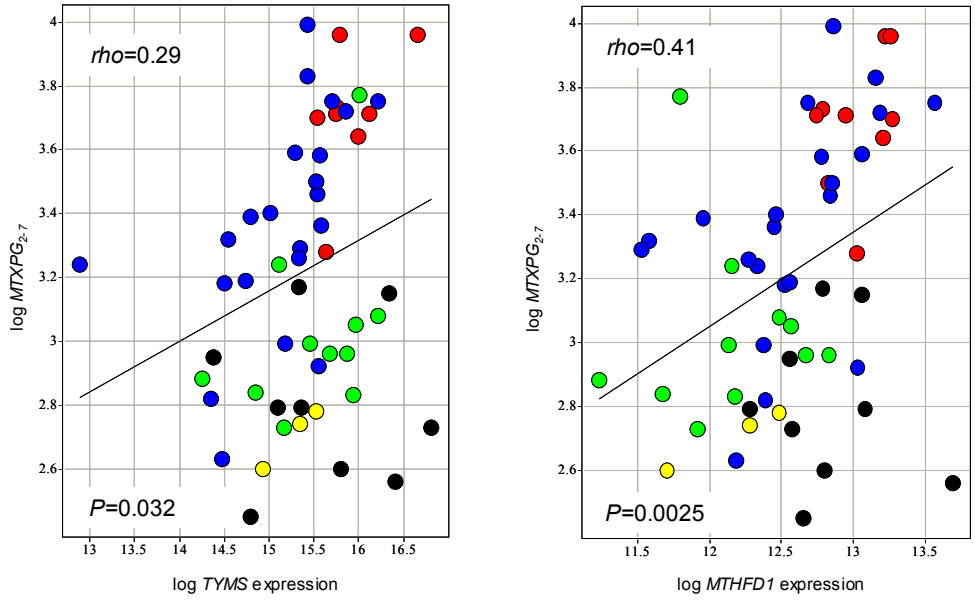
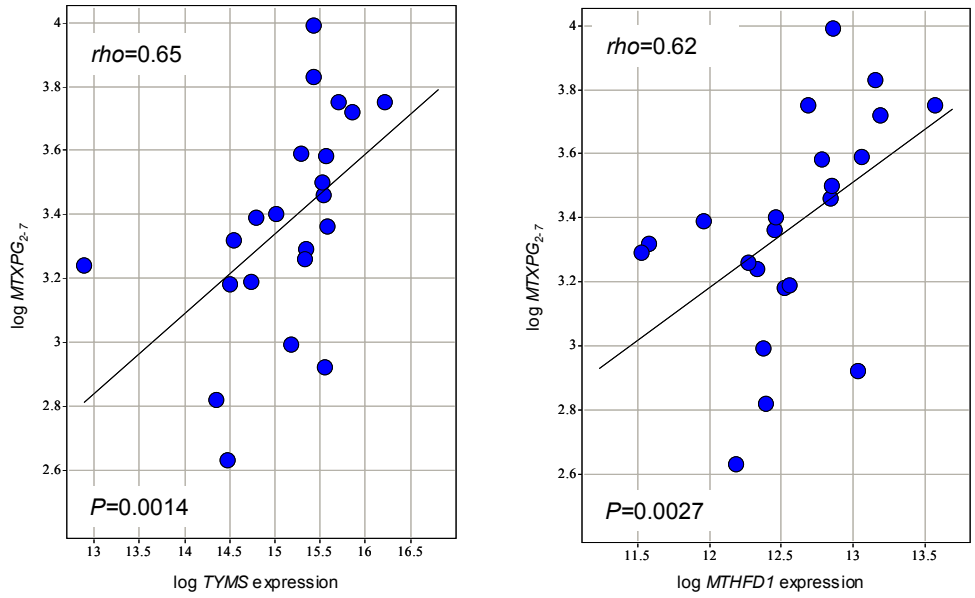


Figure S 6: Total MTXPG levels versus *TYMS* and *MTHFD1* expression in BNHD.

P-values are from Spearman correlation.



5. Correlation between S-Phase and expression of folate pathway genes

Figure S 7: Percentage S-Phase versus *DHFR* and *TYMS* expression

Data are shown for ALL cells from 52 patients. P-values are for Spearman correlation. BHD subtypes (*n*=8) are depicted in red, BNHD (*n*=18) in blue, *E2A-PBX1* (*n*=6) in yellow, T-ALL (*n*=4) in black, and *TEL-AML1* (*n*=16) in green.