Identification of *TIGAR* in the equilibrative nucleoside transporter 2-mediated response to fludarabine in chronic lymphocytic leukemia cells

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ABSTRACT

Background

The nucleoside analogue fludarabine is used in the treatment of chronic lymphocytic leukemia. It triggers p53-mediated apoptosis, although the mutational status of p53 does not fully account for heterogeneity in responsiveness to treatment. The aim of this study was to identify new genes implicated in fludarabine action as well as to determine the role of equilibrative nucleoside transporters (ENT) in the transcriptomic response triggered by this drug in chronic lymphocytic leukemia cells bearing wild type p53.

Design and Methods

We performed gene expression profiling in cells from two fludarabine-sensitive and two fludarabine-resistant cases of chronic lymphocytic leukemia treated with fludarabine either in the presence or the absence of nitrobenzylthioinosine, a hENT1-specific blocker. Twenty selected fludarabine-inducible genes were validated using Taqman low-density arrays in cells from 20 chronic lymphocytic leukemia patients with the same experimental design.

Results

Sixteen of the twenty genes (*DDB2, GADD45A, TYMS, BAX, TIGAR, FAS, TNFSF7, TNFSF9, CCNG1, CDKN1A, MDM2, SESN1, MAP4K4, PPM1D, OSBPL3* and *WIG1*) correlated with the *ex vivo* sensitivity of chronic lymphocytic leukemia cells to fludarabine, *TIGAR* (TP53-induced glycolysis and apoptosis regulator) being the gene that showed the strongest correlation ($p<0.0001$; $r^2=0.6022$). We observed that the transcriptomic response was weakly sensitive to the hENT1 blocker nitrobenzylthioinosine. Interestingly, we also found a correlation between hENT2 expression and induction of *TIGAR* after fludarabine treatment.

Conclusions

We demonstrate a correlation between the recently described p53-inducible apoptosis gene *TIGAR* and both sensitivity to fludarabine and hENT2 expression in chronic lymphocytic leukemia cells. These results, as well as the variability in fludarabine response among chronic lymphocytic leukemia patients with wild type p53, support the major role of *hENT2* in the uptake of fludarabine into chronic lymphocytic leukemia cells.

Key words: TIGAR, hENT2, fludarabine, chronic lymphocytic leukemia.

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Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults in western countries. CLL results from an accumulation of $CD5^+$ B cells due to abnormal programmed cell death.[1] The disease is characterized by a highly variable clinical course and treatment is usually restricted to patients with advanced and symptomatic disease. Historically, CLL patients have received prednisone- or chlorambucil-containing regimens, resulting in modest responses. Treatment regimens with purine nucleoside analogs, particularly fludarabine, were found to improve treatment results significantly. Therapies using fludarabine, cyclophosphamide, and/or mitoxantrone, combined with monoclonal antibodies such as rituximab or alemtuzumab result in higher overall response and complete response rates.[2]

Fludarabine is a prodrug that is converted to the free nucleoside 9-β-D-arabinosyl-2-fluoroadenine (F-ara-A), which enters cells and accumulates mainly as the 5'-triphosphate, F-ara-ATP. F-ara-ATP has multiple mechanisms of action, which are mostly directed towards DNA.[3] Several *in vitro* studies suggest that fludarabine triggers p53-mediated apoptosis in CLL cells[4,5] although cell death by p53-independent mechanisms has also been described.[6] Responses, both *in vitro* and *in vivo*, are very heterogeneous, there being reported cases with functional p53 but low response to fludarabine. The mechanisms implicated in these processes are not well known. Therefore, no suitable system to predict clinical outcome in CLL patients treated with fludarabine is available.

Anticancer therapy using nucleoside-derived analogs is dependent on drug transport across the plasma membrane and subsequent metabolic activation. Although some enzymes have been shown to be suitable biomarkers of nucleoside metabolism, thus modulating response to therapy,[7,8] the role of plasma membrane transporters in determining nucleoside-derived drug bioavailability is less well known.

The uptake of nucleosides and nucleoside-derived drugs into cells is mediated by concentrative nucleoside transporter (CNT) and equilibrative nucleoside transporter (ENT) proteins, encoded by the gene families *SLC28* and *SLC29*, respectively.[9,10] CNT proteins mediate high-affinity, concentrative nucleoside transport into cells, whereas equilibrative transporters are responsible for the facilitative uptake of nucleosides and nucleoside-derived drugs, with broad selectivity but lower affinity than that shown by CNT proteins.[11]

CLL cells express *hENT1*, *hENT2*, *hCNT2* and *hCNT3* mRNA, although most of the biological activity responsible for fludarabine uptake is associated with hENT transporters.[12] This is probably due to the fact that the hCNT3 protein is localized mostly in intracellular compartments of CLL cells,[13] while fludarabine is not a suitable permeant for hCNT2,[11] a transporter protein that is functional at the plasma membrane of CLL cells.[12] In fact, although both hENT1 and hENT2 can mediate fludarabine internalization, hENT2 but not hENT1 protein levels have recently been correlated with *ex vivo* sensitivity of CLL cells to this nucleoside analog.[14] The possibility that a single drug, despite having several transporter proteins as putative internalization pathways, channels its cytotoxic action via a particular route of entry has been recently addressed in the breast cancer cell line MCF7. In these cells, the transcriptomic response that follows treatment with 5'-deoxy-5-fluorouridine (5'-DFUR) is selectively blocked by the ENT1-specific inhibitor nitrobenzylthioinosine (NBTI),[15] even though 5'-DFUR is also a ENT2 substrate and MCF7 cells show significant ENT2-related activity.

Here we report a high-throughput analysis of gene expression in CLL cells treated *ex vivo* with fludarabine as a way to identify putative new biomarkers of fludarabine responsiveness in this disease, and we also provide evidence of transporter-mediated transcriptomic responses to nucleoside-derived drugs in primary CLL cells.

Design and Methods

Isolation and culture of primary cells

Thirty-one untreated patients diagnosed as having CLL according to the World Health Organization classification were selected on the basis of the *ex vivo* sensitivity of their cells to fludarabine (see below). Informed consent was obtained from each patient in accordance with the Institutional Ethics Committee and the Helsinki declaration. The biological characteristics of these patients are listed in Table 1. The percentage of tumor cells ($CD19^+$, $CD5^+$, and showing light-chain restriction) was analyzed by flow cytometry and ZAP-70 expression level was quantified as previously described.[16] Cytogenetic alterations were assessed by fluorescence *in situ* hybridization (FISH) using the multiprobe commercial kit from Vysis (Downers Grove, IL, USA) that contains locus-specific probes to determine the deletions of 17p (p53), 11q (ATM) and 13q and a centromeric probe to detect trisomy 12. The cut-off point for these alterations in CLL cells was >20%. p53 mutational analysis was performed according to the recommendations of the IARC TP53 consortium (*http://p53.iarc.fr*).

Peripheral blood mononuclear cells were isolated by Ficoll/Hypaque sedimentation (Seromed, Berlin, Germany) and all cases presented more than 90% tumor cells. Cells were cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide and 60% heat-inactivated fetal bovine serum (FBS, Gibco Paisley, Scotland, UK). Our previous studies demonstrated that the freezing procedure had no effect on cell response compared with that of fresh CLL cells.[17] After thawing, mononuclear cells from CLL patients were cultured in X-Vivo 10 Medium (Biowhittaker, Lonza, Belgium) at a density of 2×10^6 cells/mL, in a humidified atmosphere at 37°C containing 5% carbon dioxide.

Treatment and flow cytometry detection of apoptosis

Cells were incubated for 3, 24 and 48 hours with fludarabine 1 μg/mL (Schering, Berlin, Germany), a concentration that is comparable to that achieved in the blood during CLL treatment.[18] When indicated, cells were pre-

incubated for 1 hour with the hENT1 inhibitor nitrobenzylthioinosine (NBTI; Sigma, St Louis, MO, USA) at 100 nM before drug exposure. Cell viability was quantified by double staining with annexin-V conjugated to fluorescein isothiocyanate (FITC) and propidium iodide (PI) (BenderMedsystems, Vienna, Austria). Ten thousand cells per sample were acquired in a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and the labeled populations of cells were analyzed with the Paint-A-Gate software (Becton Dickinson).

High-density array study

Total RNA was isolated in each experimental condition by Trizol reagent (Invitrogen, Carlsbad, CA, USA), and then purified using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). In all cases the integrity of RNA was verified with a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Amplified biotinylated complementary RNA (2 μg) was produced with an *in vitro* transcription labeling reaction and was subsequently hybridized onto HU133A GeneChips (Affymetrix, Santa Clara, CA, USA), following the Affymetrix protocol for high-density arrays. Scans were carried out on an Agilent G2500A GeneArray scanner (Agilent Technologies) and the fluorescence intensities of scanned arrays were measured with the Affymetrix GeneChip software. For unsupervised clustering, dChip v1.3 software (dChip, Boston, MA, USA) was used applying a variation filter of $p<0.001$. For unsupervised analysis, scanned microarray data were introduced in the Gene Expression Pattern Analysis Suite v3.1 (GEPAS). First, genes with intensity levels below 20 were removed. After referencing each condition to its control, data were preprocessed as previously described.[19] Briefly, ratios were log-transformed (base 2) and missing values and flat patterns were filtered. The data set was then sent to a clustering tool (Sotatree) that allowed us to select the group of genes that showed different expression profiles between fludarabine-treated and fludarabine-untreated cells, and between fludarabine-sensitive and -resistant cases. In both instances, we considered only the genes whose expression differed by at least two-fold from the respective control. Functional associations between differentially expressed genes were analyzed using the Ingenuity Pathway Analysis platform (Mountain View, CA, USA).

Low-density array study

From each CLL sample, total RNA was extracted from 10^7 cells using Trizol reagent (Invitrogen). One microgram of total RNA was then retrotranscribed to cDNA using random primers with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. Forty genes were selected because of their differential expression shown in the high-density array study and five genes related to nucleoside transport and metabolism [*hCNT2*, *hCNT3*, *hENT1*, *hENT2* and *deoxycytidine kinase (DCK)*] were also included. The selected genes (*Online Supplementary Table S1*) were studied by real-time polymerase chain reaction (PCR) using TaqMan Low Density Arrays (Applied Biosystems) in an additional series of CLL patients. cDNA samples were subjected to real-time PCR in duplicate in an Abi Prism 7900HT Sequence Detection System (Applied Biosystems). The relative expression of each gene was quantified by the comparative cycle threshold (Ct) method (ΔΔCt), using β-glucuronidase (*GUS*) as an endogenous control. mRNA expression levels are given as arbitrary quantitative PCR units. For individual patient analysis, the control sample of each gene was taken as the calibrator. For comparisons among different patients, the calibrator was the average Ct value of each gene in all samples grouped together.

Table 1. Characteristics of the patients with chronic lymphocytic leukemia.

Patient n.	Age/ Gender[a]	Binet[b]	% CD19/ CD5[b]	ZAP-70[d]	p53 status[e]	Genomic alterations[f]	% sensitivity to fludarabine[g]
* CLL 1	68/M	B	98	–	wt	del13q	5
* CLL 2	54/M	B	92	+	wt	n.d.	7
* CLL 3	64/M	n.d.	97	+	wt	n.d.	7
* CLL 4	59/M	C	94	–	wt	normal	7
CLL 5	54/F	C	97	–	wt	del13q	12
* CLL 6	57/M	B	92	+	wt	del11q	15
* CLL 7	72/M	A	92	–	wt	del13q	23
* CLL 8	54/F	C	97	–	wt	del13q	34
* CLL 9	49/M	B/C	92	+	wt	del13q	36
CLL 10	78/M	A	95	–	wt	trisomy 12	36
* CLL 11	49/F	B/C	97	n.d.	wt	del11q	38
CLL 12	68/M	B	94	+	wt	normal 3	9
* CLL 13	54/M	B/C	90	n.d.	wt	trisomy 12	48
CLL 14	70/M	A	94	+	wt	trisomy 12	52
CLL 15	44/M	B/C	92	n.d.	wt	n.d.	52
CLL 16	81/M	A	93	+	wt	trisomy 12	54
* CLL 17	55/M	B/C	90	–	wt	normal	56
CLL 18	62/M	B/C	91	+	wt	normal	57
* CLL 19	46/F	B/C	94	+	wt	del13q	61
CLL 20	53/M	B	96	–	wt	normal	71
CLL 21	50/F	B/C	92	+	wt	normal	73
* CLL 22	65/M	B/C	95	+	wt	normal	75
* CLL 23	79/M	A	95	–	wt	trisomy 12	75
* CLL 24	62/M	B	91	+	wt	del13q, del11q	78
CLL 25	74/M	A	92	–	wt	trisomy 12	79
* CLL 26	58/M	B/C	90	+	wt	del11q	82
* CLL 27	73/F	B/C	90	+	wt	trisomy 12	82
* CLL 28	44/M	B/C	92	n.d.	wt	n.d.	89
CLL 29	64/M	B	90	–	wt	n.d.	92
* CLL 30	68/M	B	94	+	wt	normal	95
* CLL 31	91/F	A	91	+	wt	normal	96

**CLL cases selected for gene validation; [a]M: male; F: female; [b]according to Binet's classification; n.d: not determined; [c]quantified by flow cytometry; [d]quantified by flow cytometry; ZAP-70: +, > 20% expression; -, < 20%; n.d: not determined; [e]p53 status determined by FISH (17p deletion) and direct sequencing; wt: wild type [f]assessed by FISH. del: deletion; n.d: not determined; [g]cells were incubated with fludarabine 1 μg/mL and cytotoxicity was measured after 48 hours by annexin V staining.*

Results

Effect of hENT1 inhibition on fludarabine-induced cytotoxicity in chronic lymphocytic leukemia cells

Cells from 31 untreated CLL patients with wild type p53 were incubated with a pharmacological dose of fludarabine (1 μg/mL) and cytotoxicity was measured after 48 hours by annexin V/PI staining. As shown in

Table 1, a heterogeneous profile of sensitivity to this nucleoside analog was observed among these patients, with the relative apoptotic rate (referenced to untreated cells) ranging from 5% to 96%. Additionally, no correlation was detected between sensitivity to fludarabine and ZAP-70 expression. To determine the role of equilibrative nucleoside transporters in the cytotoxicity mediated by fludarabine, in 20 selected CLL cases (Table 1) hENT1-related transport activity was inhibited using 100 nM NBTI, as described in the *Design and Methods* section. Considering that uptake of the nucleoside analog gemcitabine is mostly mediated by hENT1[20] and previous observations suggested that this transporter isoform is a major determinant of gemcitabine-triggered cytotoxicity in patients with mantle cell lymphoma (MCL),[21] we used primary cells from a MCL patient treated with gemcitabine (25 μg/mL) as a positive control for the action of NBTI in our experimental conditions. As shown in Figure 1, NBTI completely abolished the cytotoxic effect of gemcitabine in MCL cells. In contrast, our results showed that hENT1 inhibition in CLL cells had only a slight effect on fludarabine-induced apoptosis in all cases analyzed (Figure 1), supporting the view that hENT2 contributes to fludarabine cytotoxicity in CLL cells.

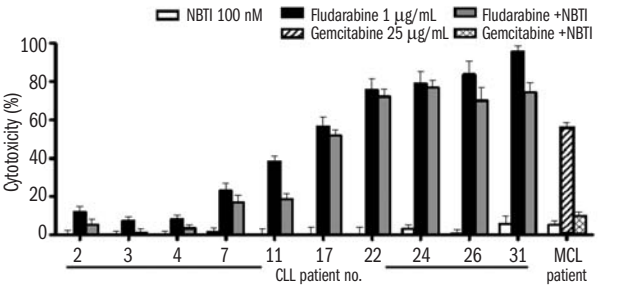

Figure 1. Effect of hENT1 inhibition on fludarabine-induced apoptosis in chronic lymphocytic leukemia primary cells. Cells from chronic lymphocytic leukemia patients were incubated with or without fludarabine (1 μg/mL) for 48 hours either in the absence or in the presence of NBTI (100 nM), added 1 hour before the fludarabine treatment. Cytotoxicity was determined by annexin-V/PI staining and results from ten representative chronic lymphocytic leukemia cases are shown. As a positive control of hENT1 inhibition, cells from a mantle cell lymphoma patient were treated for 48 hours with or without gemcitabine (25 μg/mL) in the presence or absence of NBTI (100 nM). Data (mean ± SEM) are given as percentages of the apoptotic rates relative to untreated cells.

Gene expression profile after fludarabine treatment in chronic lymphocytic leukemia patients

Primary cells from four CLL patients analyzed above with different sensitivities to the drug (CLL cases 2, 4, 24 and 26) were further used in a pharmacogenomic approach to identify genes putatively implicated in the action of fludarabine as well as to determine the role that ENT transporters may play in the transcriptomic response triggered by this drug. CLL cells (1.5×10^7) were treated *in vitro* with fludarabine (1 μg/mL) either in the presence or in the absence of NBTI (100 nM) for 3 and 24 hours. RNA was isolated from each experimental condition (untreated, NBTI, fludarabine and fludarabine+NBTI) in order to analyze gene expression profiles using the HU133A Affymetrix chip. A total of 32 chips corresponding to the 32 experimental conditions were then processed as described in the *Design and Methods* section. An unsupervised cluster analysis was performed applying a variation filter ($p<0.001$), and the resulting 2102 genes were visualized by the hierarchical clustering method (Figure 2A). Two main branches were clearly identified in the dendogram, corresponding to fludarabine-sensitive (CLL 24 and 26) and -resistant (CLL 2 and 4) CLL cases. Four secondary clusters were distinguished corresponding to each individual CLL sample, whereas small subsets also separated the two time points (3 and 24 hours) chosen for our experimental design.

Transcriptomic analysis of fludarabine-sensitive and -resistant chronic lymphocytic leukemia cells

We initially attempted to characterize genes potentially related to response to fludarabine in CLL cells. Thus, we analyzed and compared the gene expression profiles of untreated cells from the two fludarabine-sensitive (CLL 24 and CLL 26) and the two fludarabine-resistant cases (CLL 2 and CLL 4). Initial exploration of the dataset using the Preprocessor and the Clustering tools (GEPAS platform) revealed that 833 genes were differentially expressed in at least two out of the four CLL cases, and 11 clusters were identified. Two of these clusters were chosen for being either up- or down-regulated in the two sensitive cases compared to the two resistant cases (*Online Supplementary Table S2*). For all these genes, a *sensitive versus resistant* ratio index was calculated based upon the expression levels determined as the mean of the two resistant and the mean of the two sensitive CLL samples. Twenty genes in the list identified above were chosen according to the magnitude of their differential expression for further validation in a customized low-density array by real-time PCR. As shown in Figure 2B, all selected genes from the microarray analysis were validated by low-density arrays as being either up- or down-regulated in the same four CLL cases studied above. The fold-change in the *sensitive versus resistant* ratio was often different depending on the technique used, but the direction of change was always the same. All these selected genes were validated by real-time PCR in 27 CLL samples, showing different sensitivity to fludarabine (5 to 96%) (Table 1). Although in some cases tendencies were confirmed when considering the 27 CLL cases, only expression of *SERPINB9* and *SERPINE2*, both found to be over-expressed in the most fludarabine-sensitive patients (>79% fludarabine cytotoxicity) compared to the most fludarabine-resistant group (<34% fludarabine cytotoxicity) ($p<0.05$) (Figure 2C), showed a statistically significant correlation with fludarabine cytotoxicity (*SERPINB9*, p-value: 0.0182, r^2: 0.2034; *SERPINE2*, p-value: 0.0367, r^2: 0.1631). The statistical validation of only these two genes might reflect the marked heterogeneity in gene expression profiles among CLL patients. The analysis of cellular nucleoside transporters (*hCNT2, hCNT3, hENT1* and *hENT2*) and drug metabolism (*DCK*) in this set of CLL samples did not reveal any correlation between their mRNA expression levels and *ex vivo* response to fludarabine.

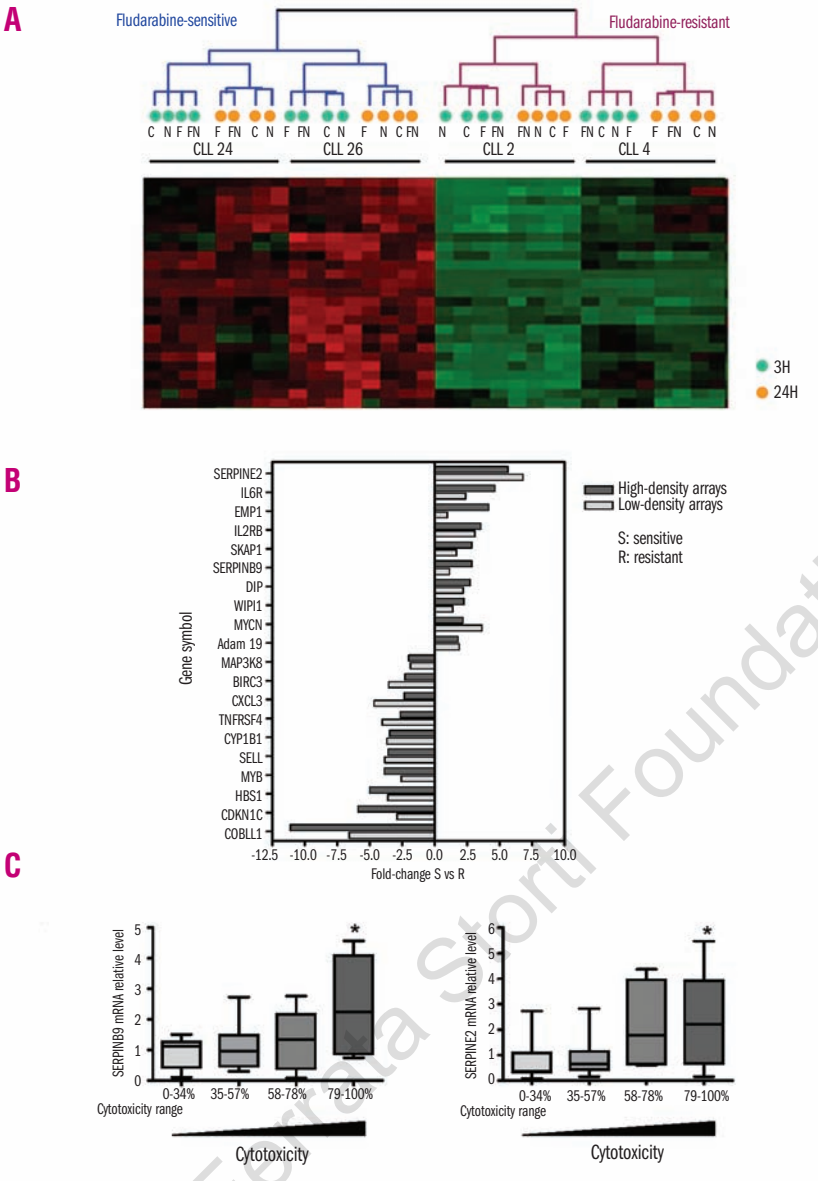

Figure 2. Gene expression analysis of fludarabine-sensitive and -resistant chronic lymphocytic leukemia cells. (A) Unsupervised hierarchical clustering of the microarray study. The 2102 genes obtained after a variation filter ($p<0.001$) were visualized by hierarchical clustering and a partial image is shown. Two main branches clearly distinguished sensitive (blue) from resistant (purple) chronic lymphocytic leukemia cases. Four clusters were identified corresponding to the four chronic lymphocytic leukemia patients included in the study, and small subsets of each cluster were constituted by the different experimental conditions from the same patient (C: control, N: NBTI, F: fludarabine, FN: fludarabine + NBTI). Red and green represent up- or down-regulation of a given gene, respectively. (B) Validation of the 20 selected genes differentially expressed in the microarray analysis performed in the same four chronic lymphocytic leukemia cases by low-density arrays. (C) Validation in the whole set of samples. The 27 chronic lymphocytic leukemia cases were distributed into four groups according to their fludarabine-response. The *SERPINB9* and *SERPINE2* genes were differentially expressed depending on fludarabine cytotoxicity. Statistical significance between the most sensitive and the most resistant group was assessed by Student's t-test (*$p<0.05$).

Genes implicated in the response of chronic lymphocytic leukemia cells to fludarabine

Changes in gene expression profiles after 3 and 24 hours of fludarabine treatment in the four selected CLL cases (CLL 2, 4, 24 and 26) were analyzed. By performing an unsupervised analysis using the preprocessor tool, we found 61 genes regulated by fludarabine after 24 hours of treatment. This analysis discriminated the two sensitive and the two resistant cases. After removing genes showing inconsistent changes and calculating the mean fold-change of those genes represented more than once on the array, we identified a set of 40 genes as being responsive to fludarabine treatment (*Online Supplementary Table S3*). Interestingly, none of these genes showed a significant change after 3 hours of treatment. A time-course analysis of the mRNA levels of selected genes (*p21, BAX, FAS* and *TIGAR*) did not show significant changes between 3 and 9 hours after treatment (*data not shown*).

Twenty fludarabine-regulated genes were chosen according to their differential expression (Table 2). These genes were validated in a customized low-density array in 20 cases of CLL. These selected cases are marked in Table 1. All but three genes (*TGFB1, Aiolos, MMP9*) were actually validated as being regulated by fludarabine (fold-change-ratio higher than 1.4). Moreover, 16 of the 20 genes showed a significant correlation between *ex vivo* sensitivity to fludarabine and gene-fold induction. These genes encoded proteins involved in the apoptotic machinery (*BAX, FAS, TNFSF7, TNFSF9* and *TIGAR*), cell cycle regulation (*cyclin G1*, *MDM2*, *p21* and *sestrin 1*), and also those encoding proteins implicated in DNA synthesis and repair (such as *thymidylate synthetase*, *DDB2* and *GADD45A*), intracellular protein trafficking (*oxysterol binding protein-like 3* and the p53 target zinc finger protein *WIG1*) and signal transduction, such as *PPM1D* and *MAP4K4*. In fact, when the mean fold-induction of the whole panel of up-regulated genes in this set of 20 CLL cases was plotted against the *ex vivo* cytotoxicity triggered by fludarabine, a highly significant correlation

Table 2. Selected fludarabine-regulated genes identified by microarray analysis and validated using low-density arrays.

Function	Gene symbol	Gene name	Fold change[a]	Correlation with sensitivity to fludarabine[b] p value	r^2	Statistical significance
DNA synthesis/repair	*DDB2*	Damage-specific DNA binding protein 2 /// LIM homeobox 3	3.17	0.0049	0.3631	**
	GADD45A	Growth arrest and DNA-damage-inducible, α	5.62	0.0069	0.3406	**
	TYMS	Thymidylate synthetase	4.89	0.0034	0.4049	**
Apoptosis/survival	*BAX*	BCL2-associated X protein	2.75	0.0012	0.4509	**
	C12orf5/TIGAR	Chromosome 12 open reading frame 5	1.87	< 0.0001	0.6022	***
	FAS	Fas (TNF receptor superfamily, member 6)	2.89	0.0008	0.4729	***
	TNFSF7	Tumor necrosis factor (ligand) superfamily, member 7	4.39	0.0027	0.4021	**
	TNFSF9	Tumor necrosis factor (ligand) superfamily, member 9	2.65	0.011	0.3084	*
Cell cycle regulation	*CCNG1*	Cyclin G1	2.06	0.0082	0.3292	**
	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	5.25	< 0.0001	0.6013	***
	MDM2	Mdm2, p53 binding protein (mouse)	8.17	0.0046	0.3682	**
	SESN1	Sestrin 1	1.4	0.0002	0.5468	***
Signal transduction	*CXCR4*	Chemokine (C-X-C motif) receptor 4	1.51	0.6184	0.015	ns
	MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4	1.64	0.0408	0.2125	*
	PPM1D	Protein phosphatase 1D magnesium-dependent, delta	2.76	0.0052	0.3592	**
	TGFB1	Transforming growth factor, β-induced, 68kDa	1.02	0.0425	0.2093	*
	ZNFN1A3/IKZF3	Zinc finger protein, subfamily 1A, 3 (Aiolos)	1.039	0.8435	0.0022	ns
Intracellular protein traffic	*MMP9*	Matrix metallopeptidase 9	0.78	0.738	0.0047	ns
	OSBPL3	Oxysterol binding protein-like 3	2.02	0.0009	0.4649	***
	WIG1/ZMAT3	p53 target zinc finger protein	2.02	0.0002	0.5388	***

[a]*Mean fold-change of fludarabine-treated cells from 20 CLL patients;* [b]*% cytotoxicity of fludarabine was correlated with gene fold-change. Statistical parameters are given (ns: non significant; * p<0.05; ** p<0.01; *** p<0.001).*

(p=0.0002, r^2: 0.55) was found (Figure 3A). It is important to note that *TIGAR*, a gene recently described as a p53-inducible regulator of apoptosis,[22] showed the strongest correlation with sensitivity to fludarabine (p<0.0001 and correlation of 0.60; Figure 3B). Functional analysis of all these probable targets of fludarabine action, using Ingenuity Pathways Analysis software, defined a p53-network of interconnecting genes, most of them (except *MMP9*) up-regulated after fludarabine treatment, which supports the biological logic of these findings (*Online Supplementary Figure S1*).

Role of equilibrative nucleoside transporters in the transcriptomic response to fludarabine in primary chronic lymphocytic leukemia cells

To elucidate the role that nucleoside transporters play in the transcriptomic response to fludarabine in primary CLL cells we performed, as discussed above, microarray analysis on cells from two fludarabine-sensitive (CLL 24 and 26) and two fludarabine-resistant (CLL 2 and 4) cases, either in the absence or in the presence of the hENT1-specific inhibitor NBTI. The magnitude of gene up-regulation (fold-induction) following fludarabine treatment in the absence of NBTI was plotted, for each patient, against the fold-induction observed under conditions of pharmacological hENT1 inhibition (incubation with NBTI). Linear regression was represented for each patient and slope values calculated (Figure 4A). A slope value close to 1 indicates that the up-regulation of these genes is not sensitive to hENT1 inhibition. This is so, for instance, for CLL 24 (slope 0.98). The other fludarabine-

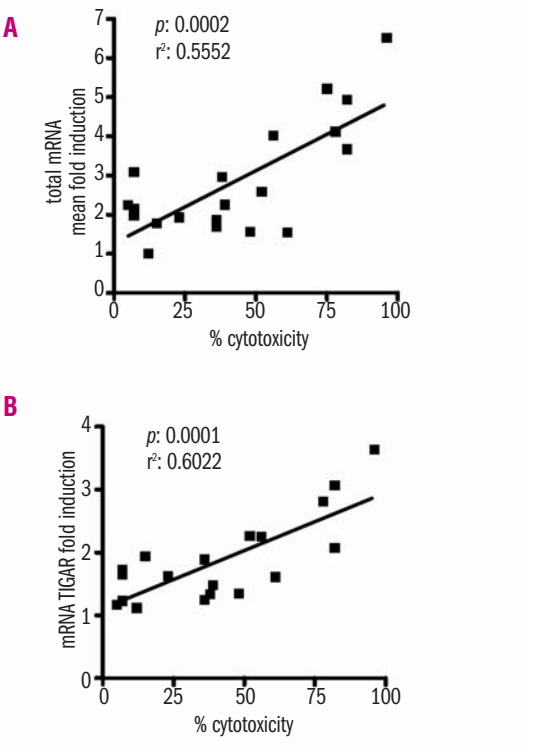

Figure 3. Genes regulated by fludarabine in chronic lymphocytic leukemia (CLL) primary cells. Cytotoxicity of cells from 20 CLL patients to fludarabine 1 μg/mL after 48 hours of treatment was plotted against the mean fold-induction of the whole panel of genes analyzed using low-density arrays (A) and the fold-induction of *TIGAR* analyzed by low-density arrays (B). Correlation coefficients and *p*-values are shown.

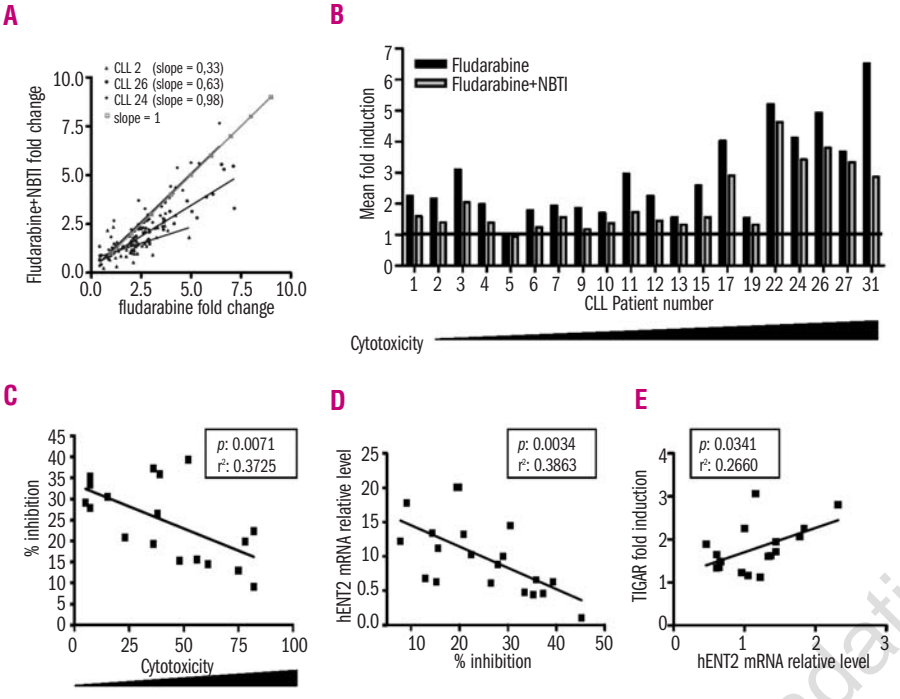

Figure 4. Role of *hENT2* in fludarabine response in chronic lymphocytic leukemia primary cells. (A) For each single case, fold-induction following fludarabine treatment (1 μg/mL) was plotted against the fold-induction after fludarabine 1 μg/mL + NBTI 100 nM treatment. Linear regression is represented for each case and slope values are shown. (B) Mean fold-change after treatment of the 20 genes analyzed using low-density arrays. Each sample is referenced to its control (untreated cells). This is shown by a horizontal line in the figure. (C) Correlation between the percentage of the gene induction inhibited by NBTI 100 nM, and cytotoxicity to fludarabine 1 μg/mL after 48 hours. (D) Correlation between the percentages of the gene induction inhibited by NBTI (100 nM) and *hENT2* mRNA level. (E) Relative *hENT2* mRNA level of cells from 20 selected chronic lymphocytic leukemia cases was plotted against the fold-induction of *TIGAR* mRNA after fludarabine treatment. Correlation coefficients and *p* values are given in the inset boxes.

sensitive case (CLL 26) also had a relatively high slope value (0.63), whereas case CLL 2, who displayed significant resistance to the action of fludarabine *ex vivo*, had a low slope value (0.33). This parameter could not be calculated for case CLL 4, since the transcriptomic response was mostly blunted, thus being completely ineffective to treatment with the hENT1 blocker, NBTI. The mean fold-induction of all up-regulated genes, quantified using the low-density arrays in the 20 CLL cases, progressively increased along with fludarabine-induced cytotoxicity. Interestingly, in any case, gene up-regulation triggered by fludarabine was completely blocked by NBTI (Figure 4B). However, the contribution of hENT1 to the transcriptomic response triggered by fludarabine was particularly relevant in case CLL 31, who showed the lowest hENT2-related mRNA levels among the whole cohort. The level of cytotoxicity induced by fludarabine was inversely correlated with hENT1 inhibition, once data from case CLL 31 were excluded (Figure 4C). In these cases the level of hENT2-related mRNA also correlated inversely with the percentage of inhibition (Figure 4D). Importantly, a significant positive correlation between hENT2 expression and induction of *TIGAR* after fludarabine treatment was also observed (p=0.0341, r^2: 0.266; Figure 4E).

Discussion

Although fludarabine is the most effective single drug for inducing a clinical response in CLL,[23] some patients do not respond to therapy. The mechanisms related to fludarabine resistance are still largely undefined. Previous studies support the idea that fludarabine is a DNA-damaging agent that increases p53 levels by promoting its post-translational stabilization, thereby inducing p53-dependent cell death.[24] Clinically, CLL patients with cells carrying *p53* gene mutations are more resistant to fludarabine regimens and have a shorter survival.[25-27] Nevertheless, other studies report that fludarabine can also induce apoptosis of CLL cells *in vitro* in a p53-independent manner.[28]

Our study was focused on CLL cases bearing a wild type *p53* genotype. Since we observed marked variability in the response of these cells to fludarabine, it is likely that other mechanisms participate in the response to this drug in CLL cells. Transcriptomic profiles of fludarabine-sensitive and -resistant CLL patients have been reported by others and most of the genes induced by fludarabine treatment turned out to be p53-dependent.[29] Our data are consistent with this view and most of the identified genes are known transcriptional targets of p53. This is illustrated by the network of interacting genes induced by fludarabine (*Online Supplementary Figure S1*). Some genes, however, were to some extent unexpectedly identified as putative mediators of fludarabine action. This is the case, for instance, for the novel p53-inducible regulator of apoptosis, *TIGAR*,[22] which was consistently up-regulated after fludarabine treatment of CLL cells, showing the highest correlation with sensitivity to fludarabine among all identified genes. *TIGAR* is the TP53-induced glycolysis and apoptosis regulator, whose up-regulation had been described after adriamycin treatment of various wild type p53 non-hematologic cell lines. Furthermore, p53-mediated upregulation of *TIGAR* resulted in inhibition of glycolysis and a decrease in cellular levels of reactive oxygen species (ROS).[22] Thus, induction of *TIGAR* after fludarabine treatment of CLL cells may explain why fludarabine induces apoptosis in a caspase-dependent manner irrespective of ROS production.[4] Our finding that fludarabine activated a p53 response in all fludarabine-sensitive cases of CLL indicat-

ed that other initial events are implicated in the response to this nucleoside analog.

Other proposed mechanisms of chemoresistance to nucleoside analogs are the dysregulation of intracellular enzymes (e.g. kinases, deaminases and nucleotidases) responsible for their metabolism,[30] or the levels of proteins implicated in drug transport.[12,31] Fludarabine is a hydrophilic compound that does not readily cross plasma membranes by diffusion; functional nucleoside transporters are, therefore, required for cellular entry. Nucleoside transporters may play a major role in facilitating nucleoside-derived drug uptake and their subsequent actions. An increasing body of evidence suggests that the amount of particular types of nucleoside transporters might determine drug-induced cytotoxicity and, accordingly, statistical correlations between the pharmacological effects of nucleoside-derived drugs and expression of nucleoside transporters have been found in a variety of types of cancer so far. Thus, the number of high-affinity binding sites for NBTI (a specific inhibitor of hENT1) has been correlated with cytarabine cytotoxicity in acute myeloid leukemia and acute lymphocytic leukemia cells,[32,33] while similar positive correlations have been reported when quantifying *hENT1*-related mRNA levels.[34-36] In fact, hENT1 protein has been reported to be a suitable biomarker for predicting survival in patients suffering from pancreatic adenocarcinoma and treated with gemcitabine (a good hENT1 substrate) as a single therapy.[37] Similarly, sensitivity to gemcitabine *ex vivo* has been reported to be positively correlated with the levels of hENT1 protein in MCL cells.[21] In fact, it has been reported that hENT1 expression, despite showing marked variability among individuals, is comparatively higher in most tumors compared to in their non-transformed tissue counterparts[38] and that hENT1 protein expression is strongly retained in gynecological tumors, particularly when compared to the highly efficient nucleoside-derived drug transporter protein hCNT1.[39]

A general consensus in the field is that hENT1 is a ubiquitous, highly expressed transporter protein that can account for most of the nucleoside supply required for salvage processes, particularly during tissue growth. Uncontrolled proliferation processes do not appear to be implicated in the development of CLL and, in accordance with what has been discussed above, hENT1 expression contributes only slightly to fludarabine-induced cytotoxicity in CLL cells,[12,14] even though fludarabine is a hENT1 substrate.[11] In fact, the levels of hENT2 protein correlate with the *ex vivo* cytotoxicity induced by this nucleoside analog in CLL cells.[12] In this study we were able to show that the transcriptomic response of CLL cells to fludarabine was poorly sensitive to nucleoside transport inhibition triggered by NBTI, a finding that is consistent with hENT2 playing a major role in the genomic response of CLL cells to fludarabine. This is relevant because it provides evidence, at the transcriptomic level, linking hENT2 function and fludarabine-triggered cytotoxicity, thus strongly suggesting that the correlation between hENT2 expression and fludarabine-induced apoptosis is not coincidental but rather the result of a drug-channeled action at the protein level, via hENT2-mediated fludarabine transport. Furthermore, it has also been described that CLL subjects with elevated hCNT3 expression, a concentrative nucleoside transporter that has been shown to mediate cellular entry of fludarabine, had a lower complete response rate to fludarabine therapy.[13] However, the current study did not show any relationship between response to fludarabine and expression of hCNT3, although it did provide evidence of a positive correlation between mRNA expression of hENT2 and induction of *TIGAR*, thus supporting the concept that hENT2-related function might determine the transcriptomic response to fludarabine.

In summary, this is the first study showing a correlation between the recently described p53-inducible apoptosis gene *TIGAR* and sensitivity to fludarabine in CLL cells. Although all patients analyzed here had wild type p53, they showed variable sensitivity to fludarabine. This finding suggests that, besides the mutational status of p53, early events in drug action, such as its transport across the plasma membrane, might determine cytotoxicity. In fact, evidence is provided of a major role of hENT2-related biological function in the transcriptomic response of CLL cells to fludarabine, a finding that strongly supports the importance of this particular nucleoside transporter in CLL chemotherapy.

Authorship and Disclosures

ML-G, LT: performed the research, analyzed the data and wrote the paper. MM-A: participated in the analysis of the data and wrote the paper. NV, FJ-C: contributed with analytical tools. EM, EC: revised the manuscript. DC, MP-A: designed and supervised the research, analyzed the data and wrote the paper. All authors revised the manuscript critically and approved the final version to be published. The authors reported no potential conflicts of interest.

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