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The pattern of gene expression and gene dose profiles of 6-Mercaptopurine- and 6-Thioguanine-resistant human leukemia cells

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ABSTRACT

Exposure of MOLT4 human T-cell leukemia cells to 6-Mercaptopurine (6-MP) and 6-Thioguanine (6-TG) resulted in acquired resistance associated with attenuated expression of the genes encoding concentrative nucleoside transporter 3 (*CNT3*) and equilibrative nucleoside transporter 2 (*ENT2*). To identify other alterations at the RNA and DNA levels associated with 6-MP- and 6-TG resistance, we compared here the patterns of gene expression and DNA copy number profiles of resistant sublines to those of the parental wild-type cells. The mRNA levels for two nucleoside transporters were down-regulated in both of the thiopurine-resistant sublines. Moreover, both of these cell lines expressed genes encoding the enzymes of purine nucleotide composition and synthesis, including adenylate kinase 3-like 1 and guanosine monophosphate synthetase at significantly lower levels than wild-type cells. In addition, expression of the mRNA for a specialized DNA polymerase, human terminal transferase encoded by the terminal deoxynucleotidyl transferase (*DNTT*) gene, was 122- and 93-fold higher in 6-TG- and 6-MP-resistant cells, respectively. The varying responses to 6-MP- and 6-TG observed here may help identify novel cellular targets and modalities of resistance to thiopurines, as well as indicating new potential approaches to individualization therapy with these drugs.

1. Introduction

The thiopurine antimetabolites 6-Mercaptopurine (6-MP) and 6-Thioguanine (6-TG) are analogues of purine nucleosides widely used to obtain and maintain remission of acute lymphoblastic leukemia (ALL) and acute myelocytic leukemia (AML) [1].

They are inactive pro-drugs that exert their cytotoxicity only after being metabolized intracellularly to products that either inhibit *de novo* purine synthesis (DNPS) or are incorporated into DNA. In the case of both 6-MP- and 6-TG, activation is catalyzed by the hypoxanthine-guanine phosphoribosyl transferase (*HGPRT*), followed by multi-step conversion to thioguanine nucleotides (TGNs) that can be incorporated into DNA or RNA and/or, with 6-MP methylated products such as methyl-thioinosine monophosphate (Me-TIMP), that inhibit *de novo* purine synthesis (DNPS) [2,3].

There is competition between such activation by *HGPRT* and deactivation through methylation of thiopurines [3] by thiopurine methyltransferase (TPMT), which is characterized by several common genetic polymorphisms [4]. It is now well-established that reduction in TPMT activity, due to genetic polymorphism results in severe and sometimes fatal hematological toxicity in patients undergoing treatment with standard doses of thiopurines and, thus, for patients with heterozygous or homozygous polymorphisms in the *TPMT* gene the dose should be lowered. On the other hand, patients with very high levels of TPMT activity may be undertreated [2].

Indeed, since cellular TPMT activity is inversely related to intracellular concentrations of TGN, patients with ALL and non-functional variant alleles of the *TPMT* genes tend to respond more favorably to 6-MP therapy, while being at higher risk of developing undesirable side-effects such as hematopoietic toxicity [4], infections, stomatitis and secondary tumors. Conversely, patients with high enzyme activity can tolerate 6-MP better but also run an increased risk for relapse and hepatic toxicity caused by methylated metabolites. To avoid life-threatening side-effects in patients homozygous for two non-functional *TPMT* alleles, a 10- to 15-fold reduction in the standard dose is recommended [3].

Inosine 5′-monophosphate dehydrogenase (IMPDH) catalyzes the first and rate-limiting step in guanine nucleotide biosynthesis. Since thioinosine monophosphate (TIMP), the major intracellular metabolite of 6-MP, is a substrate for IMPDH, alterations in the

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activity of this enzyme should exert a significant impact on thiopurine metabolism, with elevated activity promoting toxicity and attenuated activity predicting a poor clinical response [2].

In addition, guanosine monophosphate synthetase (*GMPS*), which, like IMPDH, is a key enzyme in the *de novo* biosynthesis of guanine nucleotides, catalyzes the conversion of 6-thioxanthine-5′-monophosphate (TXMP) to 6-thioguanosine-5′-monophosphate (TGMP).

Inosine triphosphate pyrophosphatase (ITPase) is another actor in thiopurine metabolism, catalyzing the reconversion of inosine triphosphate (ITP) to inosine monophosphate (IMP) [4] and thereby preventing accumulation of ITP [2]. The higher frequency of toxicity (febrile neutropenia) observed in patients with a variant non-functional ITPase allele is thought to be caused by accumulation of methylated thiopurine nucleotide metabolites, which are known to have cytotoxic properties [4].

With regards to the uptake of thiopurines, the most extensively studied proteins that transport nucleosides and nucleobases and, thus, even the thiopurines are the nucleoside transporters, which can be subdivided into two major classes: equilibrative (facilitated) transporters (the SLC29 family), and concentrative or Na^+-dependent transporters (the SLC28 family) [2].

Multi-drug resistance protein 4 (*MRP4*/*ABCC4*), which is also thought to be involved in nucleoside drug transport, has been shown recently to protect against thiopurine-induced hematopoietic toxicity by actively exporting thiopurine nucleotides. Moreover, higher levels of *MRP4* mRNA levels in the leukemia cells of pediatric patients are associated with reduced TGN levels [4].

Despite their wide-spread use, intrinsic and acquired resistance to thiopurines has become a major problem in connection with their use in chemotherapy. The mechanisms underlying the acquired resistance of leukemic cells to 6-MP- and 6-TG are still poorly understood. The most extensively characterized mechanism is a reduction or lack of *HGPRT* activity [5]. In addition, altered TPMT activity can influence sensitivity to 6-MP- and 6-TG [6]. Furthermore, in MOLT4, CCRF-CEM and Jurkat cell lines, inactivation of the mismatch repair (MMR) system leads to pronounced thiopurine resistance.

We have recently characterized two separate MOLT4 cell lines that acquired resistance to 6-MP- and 6-TG, by reducing their uptake of these drugs via the third member of the family of concentrative nucleoside transporters, *CNT3*, and the second member of the equilibrative nucleoside transporter family, *ENT2* [7]. This resistance developed in response to the classic approach, involving exposure of wild-type MOLT4 cells to stepwise increasing concentrations of the drugs.

To elucidate the mechanisms underlying acquisition of resistance to thiopurines by leukemic cells in greater details we characterized here the pattern of gene expression profiles and aberrations in DNA copy number employing high-density microarrays.

2. Materials and methods

2.1. Culturing and initial characterization of the cell lines

The generation of sublines resistant to 6-MP- and 6-TG from the acute T-lymphoblastic leukemia MOLT4 cell line has been described previously [7]. In short, the parental MOLT4 cells were exposed to 6-MP or 6-TG in gradually increasing concentrations up to 5 μM. In the present study parental and resistant sublines were cultured for at least three passages in RPMI-1640 medium supplemented with 10% FCS, 100 U penicillin/ml, 100 μg streptomycin/ml, and 2 mM L-glutamine at 37.8 °C in a humidified incubator under 5% CO_2 prior to analysis. The cells were counted using a Coulter Multisizer (Coulter Electronics, Luton, United Kingdom) and harvested during the logarithmic phase of their growth.

The expression of selected genes i.e. concentrative nucleoside transporter 1 (*CNT1*; Hs00188418_m1, *CNT2* (Hs00188407_m1), *CNT3* (Hs00223220_m1), equilibrative nucleoside transporter 1 (*ENT1*; Hs00191940_m1), *ENT2* (Hs00155426_m1), multidrug resistance-associated protein 4 (*MRP4*; Hs00195260_m1), and *MRP5* (Hs00194701_m1) by these parental and resistant cells was determined previously employing commercially available quantitative real-time PCR analysis (Applied Biosystems, Stockholm, Sweden). The mRNA levels thus obtained were related to the level of glyceraldehyde-3-phosphate dehydrogenase mRNA (*GAPDH*; Hs99999905_m1) [7].

Both parental and resistant sublines were subjected to genotyping for determination of single nucleotide polymorphism (SNP) at the Mutational Analysis Facility (MAF), Karolinska Institutet, Stockholm, using the panel of 47 markers and analysis on a Sequenom™ mass 156 spectrometer as described by Hannelius and colleagues [8]. Pair-wise comparison revealed identical genotypes in parental and 6-MP-resistant cells and 88% identity between parental and 6-TG-resistant cells.

2.2. RNA extraction

Total RNA was extracted from cultured cells using the RNeasy Midi kit and the protocol recommended by the manufacturer (RNeasy Midi Handbook; Qiagen, KEBO Lab, Spånga, Sweden). RNA concentrations and quality were determined using a NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE), with extracts exhibiting absorbance on ratio of 1.99–2.0 at 260/280 nm being regarded as of acceptable purity.

2.3. Microarray evaluation of gene expression

The oligonucleotide microarrays and all other reagents required for these analysis were purchased from Affymetrix (Affymetrix Inc., Santa Clara, CA, USA). The wild-type, 6-TG-resistant and 6-MP-resistant MOLT4 cell lines were analyzed in triplicate in accordance with the manufacturer's instructions (Technical manual of Affymetrix GeneChip products). The experimental and analysis procedures have also been described in detail in a previous publication from our laboratory [9]. The data were analyzed using the GeneSpring software (Agilent) and subsequently categorized with the Ingenuity software for normalization and exclusion of probe sets that did not meet criteria for detection. Probe sets with low expression below 50 in intensity value were excluded. Statistical calculations were performed by Analysis of variance (ANOVA) with $p < 0.05$ as the cut-off for statistical significance. Selective functional categorization of differentially expressed genes was done using Ingenuity software. All of these microarray data are available at http://www.ncbi.nlm.nih.gov/geo/.

2.4. DNA isolation and array comparative genomic hybridization (array-CGH)

Array-CGH was carried out and analyzed essentially as described previously [10]. Genomic DNA was extracted using the GenElute™ kit (Sigma–Aldrich, Inc.) and quantified and subjected to quality control by NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Cellular DNA labeled with Cy3-dCTP and reference DNA (Promega, USA) labeled with Cy5-dCTP were pooled, mixed with human Cot-1 DNA, and hybridized to tiling 38 K BAC arrays (SCIBLU Genomics Centre at Lund University, Sweden; www.lu.se/sciblu) for 72 h at 37 °C. After washing and drying, the slides were scanned