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The gene expression profiles of medulloblastoma cell lines resistant to preactivated cyclophosphamide

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Abstract

The total expression profiles of two medulloblastoma cell lines resistant to the preactivated form of cyclophosphamide (4-hydroperoxycyclophosphamide, 4-HC) were examined using the Affymetrix GeneChip U133A array. Our primary objective was to look for possible genes, other than the well-studied aldehyde dehydrogenases (ALDH) that may be involved in cyclophosphamide (CP) resistance in medulloblastomas. We present here the lists of the most highly upregulated [30 for D341 MED (4-HCR); 20 for D283 MED (4-HCR)] and downregulated [19 for D341 MED (4-HCR); 15 for D283 MED (4-HCR)] genes which may be involved in conferring CP-resistance to the two medulloblastoma cell lines. The lists of genes from the two sublines almost had no overlap, suggesting different mechanisms of CP-resistance. One of the most noteworthy upregulated gene is TAP1 [90-fold increase in D341 MED (4-HCR) relative to D341 MED]. TAP1, a protein belonging to the ABC transporter family is normally involved in major histocompatibility class I (MHC I) antigen processing. This suggests the possible role of multidrug resistance (MDR), albeit atypical (which means it does not involve the usual MDR1 and MRP glycoproteins), in medulloblastoma's CP-resistance. Apart from TAP1, a number of other genes involved in MHC1 processing were upregulated in D341 MED (4HCR). D341 MED (4-HCR) also had a 20-fold increase in the expression of the aldo-keto reductase gene, AKR1B10, which may deactivate the reactive cyclophosphamide metabolite, aldophosphamide. For D283 MED (4-HCR), the most notable increase in expression is that of ALDH1B1, a member of the aldehyde dehydrogenase (ALDH) family of proteins.

Keywords

cyclophosphamide; drug resistance; medulloblastoma; brain tumor; oxazaphosphorine; aldehyde dehydrogenase; microarray; aldo-keto reductase

Introduction

Cyclophosphamide (CP), the DNA alkylating drug of the oxazaphosphorine family, is widely-used to treat a variety of hematological cancers and solid tumors [reviewed in [1]], including the pediatric neuroectodermal tumor medulloblastoma[2, 3]. To be active, CP requires that mixed function oxidases in the liver convert the drug to 4-hydroxycyclophosphamide (4OH-CP) [4]. In solution, 4OH-CP is in equilibrium with its

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acyclic tautomer, aldophosphamide [5]. Aldophosphamide eventually converts to the reactive metabolite, phosphoramidate mustard (PM), which directly alkylates and crosslinks DNA strands, eventually leading to cell death [6–8]. As in many chemotherapeutic drugs, a major hindrance to the effectiveness of CP in long term cancer treatment is the target cells' subsequent development of resistance against the drug. In order to understand the molecular mechanisms behind CP resistance in medulloblastomas, our laboratory has established the 4-hydroperoxycyclophosphamide (4-HC)-resistant medulloblastoma sublines D283 MED (4-HCR) and D341 MED (4-HCR) [9], derived from the parental cell lines D283 MED [10] and D341 MED [11] respectively. 4-HC, a preactivated form of CP, spontaneously degrades to the reactive 4-OH-CP in physiological conditions [12]. Our earlier investigations showed that both of these 4-HCR sublines have elevated levels of GSH [9, 13]. D283 MED (4-HCR) also had an elevated aldehyde dehydrogenase (ALDH) activity and was capable of repairing DNA interstrand crosslinks (ICLs) [14]. Despite the knowledge gained from previous studies, investigators in the field acknowledge the fact that cancer drug resistance can be very complicated, oftentimes involving multiple factors. To accelerate our search for other candidate genes that may be involved in the development of CP-resistant phenotypes in medulloblastomas, we employed the use of Affymetrix Human Genome U133A arrays. This approach resulted into a number of candidate genes that may be contributing factors to how CP and other oxazaphosphorines are resisted by medulloblastomas, and possibly other types of cancer.

Materials and Methods

Cells

The generation and maintenance of D283 MED (4-HCR) and D341 MED (4-HCR) as well as corresponding parental lines (D283 MED, D341 MED) have been described previously [9–11].

Microarray Experiment

Total RNA from D-341 MED, D-341 MED (4-HCR), D283 MED, and D283 MED (4-HCR) cells were extracted (in triplicates, three independent cell cultures) using Trizol Reagent (Life Technologies) and purified with RNeasy columns (Qiagen). Double stranded cDNA was synthesized from 10 µg total RNA using cDNA synthesis kit (Life Technologies), and T7 (dT)24 primer (GenSet). Using the BioArray High Yield RNA Transcription Kit (with T7 RNA polymerase) from Enzo Diagnostics (New York, NY), the cDNA was transcribed in vitro to produce biotin-labeled cRNA. The cRNA was then hybridized to GeneChip Human Genome U133A Array according to the Affymetrix protocol (www.affymetrix.com). All microarray experiments were done in triplicates, and data collections followed the MIAME (minimum information about microarray experiment) guideline [15].

Statistical Analysis

Affymetrix intensity (CEL) files were normalized and quantified using Bioconductor [16] with the RMA (robust multi-array average) model [17]. Differentially expressed genes were identified with an empirical Bayesian model, which shrunk the estimated sample variance towards a pooled estimate [18]. This approach is more robust whenever the number of arrays is small. Hybridization intensities were visualized using the linear rainbow color scale [19].

Real Time PCR

Total RNA from all parental and 4-HCR lines were extracted as detailed in the Microarray Experiment section. Primers for the PCR reactions (Table 1) were all designed using the web-based Primer3 software (<http://frodo.wi.mit.edu/>). In a 10 µL reaction volume, 1 µg of

total RNA was reverse transcribed using the 12.5 units of MultiScribe Reverse Transcriptase, 2.5 μ M random hexamers and all the recommended components (Applied Biosystems Inc.). The generated cDNA was diluted 1:50 and 5 μ L of which was mixed with 12.5 μ L SYBR Green PCR Master Mix, 2 μ L each of 10 μ M forward and reverse PCR primers, and the reaction volume adjusted to 25 μ L. All the PCR reactions (in 96-well plates) were run in the ABI Prism 7900HT Sequence Detection System (SDS). Conditions were as follows: a) stage 1 (50 °C for 2 min) b) stage 2 (95 °C for 10 min), stage 3 (40 cycles of 95 °C for 15 sec, and 60 °C for 1 min), and stage 4 (95 °C, 60 °C, 95 °C at 15 sec each in that order), followed by cooling at 4 °C. Analysis of results was done using the ABI Prism 7900 HT SDS software.

Results

Statistical Analysis of Microarray Data

We compared the expressions of both 4-HCR sublines from its corresponding parental lines. Illustrated in Fig. 1 are the most upregulated and downregulated genes in D341 MED (4-HCR) (Fig. 1a) and D283 MED (4-HCR) (Fig. 1b) relative to their parental lines. There is virtually no overlap between these gene lists, suggesting that these two 4-HCR lines have distinct drug resistance mechanisms.

A) D341 MED (4-HCR)—The most notable gene with upregulated expression in D341 MED (4-HCR) is AKR1B10 (aldo-keto reductase family 1, member B10 (aldose reductase)), which belongs to a family of proteins known to be involved in oxazaphosphorine resistance [5]. Also noted was the increased expression of TAP1 (transporter 1, ATP-binding cassette, sub-family B), a member of a subfamily of ATP-binding cassette (ABC) transporters, which also includes the multi-drug resistant gene MDR1 (reviewed in [20]). The higher mRNA levels of AKR1B10 and TAP1 were confirmed by quantitative real time PCR (Table 2). Other upregulated genes in D341MED (4-HCR) were the Major Histocompatibility Complex (MHC) Class I genes HLA-A, HLA-G, HLA-B, HLA-C, and B2M (beta-2-microglobulin). Quantitative PCR also verified the overexpression of HLA-B and B2M (beta-2-microglobulin) in D341 MED (4-HCR), relative to its parental line. Also evident from microarray data analysis was the upregulation of two subunits of the proteasome (PSMB8 and PSMB9), both of which are also crucial in the processing of MHC Class I peptides [21]. Other very highly upregulated genes were PEG10 (paternally expressed 10), NEF3 (neurofilament 3 (150kDa medium)), GBP1 (guanylate binding protein 1, interferon-inducible, 67kDa), STAT1 (signal transducer and activator of transcription 1, 91kDa), GJA1 (gap junction protein, alpha 1, 43kDa (connexin 43)), CHGB (chromogranin B (secretogranin 1)), ID2 (inhibitor of DNA binding 2, dominant negative helix-loop-helix protein), and SSX2 (synovial sarcoma, X breakpoint 2), all with at least 6-fold increase in the drug resistant subline compared to the parental cell line. On the other hand, among the most downregulated genes were RPS4Y1 (ribosomal protein S4, Y-linked), LMO2 (LIM domain only 2 (rhombotin-like 1)), PROM1 (prominin-like 1 (mouse)), NFIB (nuclear factor I/B), DRD1 (dopamine receptor D1), SPON1 (spondin 1, extracellular matrix protein), and NRN1 (neuritin 1).

B) D283 MED (4-HCR)—Not surprisingly, we were able to identify the elevated expression of an aldehyde dehydrogenase gene (ALDH1B1, aldehyde dehydrogenase 1 family, member B1) in the D283 MED (4-HCR) subline. The rest of upregulated genes include: MAGEA10 (melanoma antigen family A, 10), PDE6A (phosphodiesterase 6A), OLFM1 (olfactomedin related ER localized protein; pancortin 1), LMO3 (LIM domain only 3 (rhombotin-like 2), the gene for proteolipid CD9 antigen, NCAM1 (neural cell adhesion molecule 1), and PTPN13 (protein tyrosine phosphatase, non-receptor type 13 (APO-1/

CD95 (Fas)-associated phosphatase). Using Real Time PCR, we were able to validate the upregulation of ALDH1, LMO3, and MAGEA10, with the latter showing only marginal RNA level increase in the drug resistant subline (Table 2). Among the most downregulated genes were a number of GAGE genes, MARCKS (myristoylated alanine-rich protein kinase C substrate), PCDH8 (protocadherin 8), COL4A6 (collagen, type IV, alpha 6), and SPARC (secreted protein, acidic, cysteine-rich (osteonectin)).

Discussion and Conclusion

A number of factors have been known to reduce the chemotherapeutic activity of cyclophosphamide (CP) [22]. Well-understood is CP's metabolic deactivation by aldehyde dehydrogenases [1, 23, 24]. The role of Glutathione (GSH)/ Glutathione-S-transferase (GST) system in CP-resistance is still not clear as results from several studies were contradictory [25, 26]. Our group has previously shown that depletion of MGMT (O^6 -methylguanine-DNA methyltransferase) by O^6 -benzylguanine (O^6 -BG) in the D283 MED (4-HCR) subline can improve 4-HC's activity [27]. In that paper, it was proposed that MGMT is involved in repairing the O^6 -guanine acrolein (a by-product of CP metabolism) adduct, implying that acrolein is also important in CP cytotoxicity. The inverse correlation between MGMT activity and CP's toxicity in lung cancer cells has also been reported [28]. In addition, our group previously demonstrated that D283 MED (4-HCR)'s DNA interstrand crosslink (ICL) repair activity can also diminish CP cytotoxicity [14]. Despite the well-defined mechanisms of CP-resistance in cancer, we believe that transcriptional profiling of CP-resistant cell lines could still help decipher additional clues on why many cancer patients still fail CP-therapy. Moreover, the problem of CP-resistance is most probably tissue-specific. In this case, we only focused on model cell lines for pediatric medulloblastoma.

In the D341 MED (4-HCR) line, we identified the overexpression of TAP1, which along with TAP2, forms the functional TAP. The TAP heterodimer belongs to the ATP-binding cassette (ABC) transporter superfamily of proteins which also include MDR1 (p-glycoprotein) and the MDR-related protein (MRP) family [20]. Lage *et al* has presented evidence associating TAP overexpression to a gastric carcinoma cell line's resistance to the DNA-intercalating drug mitoxantrone [29]. As in the case of our 4-HCR sublines, they did not detect the elevated mRNA levels for TAP2 in the mitoxantrone-resistant line. However, the difference in TAP2 expression between the parental and drug-resistant lines was clearly evident in their Western blot. Not the typical multidrug-resistant (MDR) protein, TAP is normally involved in the processing and transport of cytosolic peptides for presentation to T cells as antigens bound to class I major histocompatibility complex (MHC I) protein [30]. Lage *et al* hypothesized that TAP can get rid of the mitoxantrone by translocating the drug from cytosol into the ER lumen. Incidentally, TAP1 is located in the same chromosomal region (6p21.3) as the MHC proteins (HLA-A, B, C, and G), and the proteasome proteins PSMB 8 and 9 (also involved in Class I MHC processing), all of which have increased expressions in D341 MED (4-HCR). In a study of another cell line with mitoxantrone resistance, the 6p21.3 region is marked by chromosomal gain as shown by comparative genomic hybridization [31], thus the higher mRNA levels of these MHC and MHC-related genes may be due to DNA amplification in this specific chromosomal region. However, another class I MHC protein, beta 2 microglobulin (B2M), whose locus is found in a different chromosome (15q21-q22.2), is also upregulated. Previous studies have also demonstrated higher expression levels of MHC molecules, such as B2M, in multi-drug resistant tumor cells [32]. It should also be noted that the expression of many of the genes in the MHC I locus can be induced by IFN gamma [33, 34]. The guanylate binding protein 1 (GBP1), as well as STAT1 (signal transducer and activator of transcription 1, 91kDa), both of which were overexpressed in D341 MED (4-HCR) are also inducible by IFN gamma [33]. It is possible that a disruption in the IFN gamma signaling pathway (which involves

the JAK-STAT pathway) may have caused the constitutive expressions of these genes (Table 3) including TAP in D341 MED (4-HCR). Whether all these MHC-1 proteins are directly involved in drug resistance can only be answered through additional experiments.

D341 MED (4-HCR) also exhibited increased AKR1B10 (aldo-keto reductase family 1, member B10, aldose reductase) expression (20-fold relative to the parental line). Aldose reductases have been shown *in vitro* to catalyze the reduction (thus inactivation) of aldophosphamide to alcophosphamide [35–37], thus this superfamily of proteins has long been recognized as important factors in CP and oxazaphosphorine-resistance [5]. To our knowledge, this would be the first report of upregulation of an aldose reductase gene in CP-resistant cancer cells. AKR1B10 has also been shown to efficiently reduce aliphatic and aromatic aldehydes [38]; aldophosphamide can be classified into the latter category. Another interesting gene which increased its expression in D341 MED (4-HCR) is PEG 10 (paternally expressed 10). In hepatocellular carcinoma cells, its overexpression decreased cell death mediated by SIAH1 (seven in absentia homolog 1 (Drosophila)) [39]. GJA1 (gap junction protein, alpha 1, 43kDa (connexin 43)), also with elevated RNA level in D341 MED (4-HCR), was upregulated in cisplatin-resistant ovarian cancer cells [40]. However, the authors found that inhibition of connexin 43 function in such cells actually led to increase in drug resistance [40].

Among the downregulated genes in D341 MED (4-HCR), one of the most noteworthy is PEG3. This protein has been shown to promote apoptosis by inducing the translocation of Bax from cytosol to mitochondria [41]. Its decreased expression may then contribute to the survival of the drug-resistant cells. BIN1 (bridging integrator 1), also downregulated in D341 MED (4-HCR), is a MYC-interacting tumor suppressor, whose reduced expression was seen in various types of cancer [42]. Another gene which exhibited lower expression level in D341 MED (4-HCR) is TIMP3 (tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)), which was also found to be one of the most downregulated genes in ovarian cancer tissue samples taken after chemotherapy, leading the authors to suspect that the reduced expression of this gene is a signature of chemotherapy resistance [43].

The higher expression level of ALDH1B1 in D283 MED (4-HCR) relative to its parental line is not exactly surprising since aldehyde dehydrogenases (ALDHs) have been known to detoxify oxazaphosphorines. ALDH can oxidize aldophosphamide into carboxyphosphamide, preventing the formation of the reactive phosphoramidate mustard [5]. Our previous study has already shown that D283 MED (4-HCR) registered an ALDH activity not detected in its parental line [13]. Among the ALDH family of proteins, the levels of ALDH1A1 and ALDH3A1 have been shown to correlate with survival of cancer cells to oxazaphosphorine treatment [23, 44]. Whether ALDH1B1 is also important in the detoxification of CP, as suggested by the results of this study, deserves further investigations. Other genes that have increased expressions in D283 MED (4-HCR), and subsequently verified by real time PCR are the presumed transcription factor LMO3 (LIM domain only 3 (rhombotin-like 2)), and the X chromosome gene MAGEA10 (melanoma antigen family A, 10). LMO3 has been shown to act like an oncogene while interacting with the neural transcription factor HEN2 in neuroblastoma [45]. The poor prognosis of primary neuroblastomas also coincided with LMO3's upregulation. On the other hand, MAGEA10, is a member of the MAGE family of genes, which have been considered as identifiable markers in melanomas and hepatocarcinomas [46, 47]. Another potentially important gene which was upregulated in D283 MED (4-HCR) is PTPN13 (protein tyrosine phosphatase, non-receptor type 13 (APO-1/CD95 (Fas)-associated phosphatase)), which has been shown to negatively regulate Fas-mediated apoptosis [48]. Our microarray analysis also revealed the increase in expression NCAM1 (neural cell adhesion molecule 1, or CD56) in D283

MED (4-HCR). In acute myeloid leukemia (AML), CD56 expression among AML patients with t(8:21) translocation has been shown to correlate to lower disease-free survival [49]. Another highly downregulated gene in D283 MED (4-HCR) is SPARC (secreted protein, acidic, cysteine-rich (osteonectin)). In an earlier study, it was shown that re-expression of SPARC was able to restore a colon cancer cell line's sensitivity to 5-fluorouracil and the topoisomerase I inhibitor, irinotecan [50]. We also observed the downregulation of a number of Cancer/Testis (CT) antigens such as GAGE1, 3, 7, and 12C. However, it is not exactly clear why these results are contrary to what most investigators observed regarding these cancer/testis genes. CT genes are oftentimes found to be overexpressed in different types of cancer, raising their potential as antigenic targets for therapy and diagnosis (reviewed in [51]). The overexpression of a number of GAGE genes was also detected in paclitaxel-resistant cell lines [52].

The upregulation of ALDH1B is consistent with our previously reported higher ALDH activity in D283 MED (4-HCR) [13] (See Table 4). Although we previously observed the increased level of GSH in both drug-resistant sublines, our microarray analysis did not reveal the upregulation of genes involved in glutathione biosynthesis, such as γ -glutamylcysteine synthetase (GCS). However, aside from upregulation of GCS, other factors such as cysteine levels can affect GSH synthesis (reviewed in [53]). We have also identified MGMT as a possible contributing factor to CP-resistance in D283 MED (4-HCR). However, in our microarray analysis, we did not observe the upregulation of MGMT in the same way that higher MGMT activity and protein levels [54, 55], as well as mRNA levels (unpublished results from microarray analysis) were easily detected in the BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea)-resistant sublines of D283 MED. The primary reason for this could be the fact that it is more crucial for D283 MED cells to have MGMT repair the O^6 -guanine chloroethyl lesion (caused by BCNU) [56] than to have MGMT repair the O^6 -guanine acrolein adduct (caused by CP). The presence of DNA interstrand crosslink (ICL) activity in D283 MED (4-HCR) [14] is also not readily explained by the results of our microarray analysis, as we did not see the upregulation of any possible gene that may be involved in DNA interstrand crosslink repair [57]. This process, however involves numerous genes and that regulation at protein level can be just as important as regulation at transcriptional level.

Although some of the upregulated genes in our HC-resistant medulloblastoma sublines belong to family of genes known to directly affect CP-metabolism (such as ALDH1B and AKR1B10), it would be interesting to find out if changes in the expression levels of these genes and others in the candidate list (Figure 1, Supplement Tables 1 and 2) will actually influence the outcome of CP therapy. An ideal experimental model would be to analyze the transcriptomes of biopsy samples obtained during the course of treatment from a regimen involving CP, then relate the data to clinical results. In reality, however, most biopsies are taken prior to treatment, thus the analysis of the expression profiles of such samples would miss important genes whose upregulation or downregulation during the course of treatment may affect the effectiveness of the therapy. Nonetheless, a couple of studies have analyzed the genome-wide expression levels of medulloblastoma biopsies collected prior to treatment. In the study by Pomeroy *et al* [58] which involved pre-treatment biopsies of 60 medulloblastoma patients, they observed that elevated levels of certain genes (including a number of cell proliferation genes, as well as the multidrug resistance gene *sorcin*), are more common among tumors of patients who responded poorly to a chemotherapy regimen which included CP. In a similar study involving 35 newly diagnosed medulloblastoma patients, Neben *et al* [59] also identified a number of genes whose raised levels of expression negatively correlate to patient survival. Interestingly, the list included ALDH1A (an isozyme of aldehyde dehydrogenase), even though the chemotherapy regimen comprised of lomustine, cisplatin, and vincristine, none of which belong to the oxazaphosphorine family

of drugs. Park and co-workers also conducted a genome-wide transcriptional analysis of pre-treatment biopsies of breast cancer patients who were eventually treated with a regimen which included CP [60]. The authors found out that a number of ABC transporter genes were elevated in patients who exhibited residual residue of the disease compared to those who were classified to have pathologic complete response from the treatment. In an era when new generations of drugs (those already used in clinics, as well as those still in clinical trials) are designed to target specific proteins or family of proteins involved in cancer proliferation, genome wide expression analysis of biopsies has the potential to help direct the course of treatment for a specific patient. In terms of discovering genes/gene mutations that directly affect the effectiveness of cancer drugs, the use of model cell lines induced for resistance to drugs (through constant exposure) can still provide very important information that may not be discovered in most biopsy studies. In recent years, the approach of inducing a cancer cell line for resistance to a certain drug, and subsequently profiling its genome wide expression, in the hope of discovering cancer drug resistance genes (which can also be tissue specific aside from being drug specific), has been employed by a number of laboratories. These most recent publications include the studies of breast cancer [61], oral carcinoma [62], and ovarian carcinoma [63] cell lines resistant to cisplatin, a breast cancer cell line resistant to tamoxifen [64], and leukemia cell line resistant to imatinib [65].

Through the use of these medulloblastoma cell lines induced for resistance to CP, and their subsequent genome wide expression profiling, we presented here a list of genes whose expression level changes may mirror the situations when medulloblastoma patients start to develop resistance to CP during the course of treatment. In particular, the upregulation of TAP1, AKR1B10, and a particular isoform of aldehyde dehydrogenase (ALDH1B1) suggests the possible importance of these genes as markers of CP-resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression heat map depicting the most highly upregulated and downregulated genes in D341 MED (4-HCR) (A) and D283 MED (4-HCR) (B) relative to their parental cell lines (D341 MED and D283 MED respectively). The expression array analysis for each cell line was run in triplicate (3 distinct heat map columns per cell line). Also shown are the probesets and gene IDs corresponding to rows in the heat maps. The expression levels of the genes marked in red were verified by Real Time PCR. (The genes are also listed in Supplement Tables 1A and B, 2A and B).

Table 1

The nucleotide sequence of the primers used for quantitative real time PCR validation of the raised levels of expressions of select genes in the two 4-hydroperoxycyclophosphamide-resistant (4-HCR) medulloblastoma cell lines

Gene	Forward Primer	Reverse Primer
ACTB	5'-AGA AAA TCT GGC ACC ACA CC	5'-TGG GGT GTT GAA GGT CTC A
AKR1B10	5'-TTC AGC CAC TTC CAG ATC G	5'-CTG TAG GCC GTA ACG GTG AT
ALDH1B1	5'-CAG ATC ATC CCG TGG AAC TT	5'-GCC AAA TAC AGG GCA GAG AG
HLAB	5'-GAG GAA GAG CTC AGG TGG AA	5'-TGC ATC TCA GTC CCT CAC AA
B2M	5'-TGA CTT TGT CAC AGC CCA AG	5'-AGC AAG CAA GCA GAA TTT GG
TAP1	5'-TCC GGA AAC CGT GTG TAC T	5'-AGA AGC ACT GAG CGG GAG TA
MAGEA10	5'-GGG AGA GCA AGA GGT CAA GA	5'-CAG GGT AGC AGG TGG GAG T
LMO3	5'-CGT GCC AAG GAC AAT GTT TA	5'-CCT TCC TCG TAG TCC GTC TG

Table 2

Verification of select upregulated genes in the two drug resistant sublines by real time PCR. The housekeeping gene beta actin (ACTB1) was used as control.

Cell Lines Being Compared	Gene	ΔC_t^a	Expression Ratio ^b
D341 MED (HCR) vs D341 MED	<i>ACTB</i> (reference)	-0.64	
	<i>TAP1</i>	-7.18	93.2
	<i>AKR1B10</i>	-4.99	20.4
	<i>B2M</i>	-5.71	33.6
	<i>HLAB</i>	-7.81	144.6
D283 MED (HCR) vs D283 MED	<i>ACTB</i> (reference)	-0.05	
	<i>ALDH1B1</i>	-3.53	21.9
	<i>LMO3</i>	-4.50	11.2
	<i>MAGEA10</i>	-1.40	2.5

^a ΔC_t = Average C_{tHCR} – Average $C_{tParent}$

^b Expression Ratio = $2^{-[\Delta C_t (\text{target gene}) - \Delta C_t (\text{ACTB})]}$

Table 3

Known IFN-Gamma- induced genes which are also overexpressed in D341 MED (4-HCR). These are also among the list of genes discussed in a review by Boehm et al [33]

Gene Symbol	Gene Name	Chromosomal Location
HLA-A	major histocompatibility complex, class I, A	6p21.3
HLA-B	major histocompatibility complex, class I, B	6p21.3
HLA-C	major histocompatibility complex, class I, C	6p21.3
HLA-G	HLA-G histocompatibility antigen, class I, G	6p21.3
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	6p21.3
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional protease 2)	6p21.3
PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional protease 7)	6p21.3
B2M	beta-2-microglobulin	15q21-q22.2
STAT1	signal transducer and activator of transcription 1, 91kDa	2q32.2
GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	1p22.2

Table 4

The previously observed and some of the microarray expression analysis-suggested cyclophosphamide resistance factors in D283 MED (4-HCR) and D341 MED (4-HCR) sublines

4-HCR medulloblastoma subline	Previously shown mechanisms (Refs)	Some of the upregulated genes detected by microarray analysis
D341 MED (4-HCR)	1 GSH [13] (Friedman <i>et al.</i> , 2002)	1 TAP1 2 AKR1B10
D283 MED (4-HCR)	1 MGMT (Friedman <i>et al.</i> , 1999) 2 ALDH (Friedman <i>et al.</i> , 2002) 3 GSH (Friedman <i>et al.</i> , 2002) 4 DNA interstrand crosslink repair (Dong <i>et al.</i> , 1996)	1 ALDH1B1