Response of Medulloblastoma Cells to Vincristine and Lomustine: Role of TRKC, CTNNB1 and STK15

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Abstract. *Background: Vincristine and lomustine are two important chemotherapeutic drugs used for the treatment of different types of neoplasms, including medulloblastomas. Materials and Methods: We investigated the effects of vincristine and lomustine on 12 primary medulloblastoma cell cultures and the DAOY cell line using the annexinV-flow cytometry and immunoblotting techniques, following treatment of cells for different periods of time. Results: Both drugs triggered apoptosis and cell cycle delay at the G_2/M phase and also up-regulated p16. Furthermore, the expression of 8 different cancer-related genes were assessed and their mRNA and protein levels were found to be highly heterogeneous and did not correlate in several medulloblastoma cultures. Importantly, there was significant correlation between the level of cadherin-associated protein beta 1 (CTNNB1) and Aurora kinase A (STK15) proteins and neurotrophic tyrosine kinase receptor type 3 (TRKC) mRNA and the proportion of apoptosis induced by vincristine, the combination of both drugs, and lomustine, respectively. Conclusion: These genes could be of great importance as therapeutic biomarkers during the treatment of medulloblastoma patients with vincristine and lomustine.*

Medulloblastoma, aggressive tumor of the cerebellum, is the most common malignant brain tumor in children. It represents approximately 20% of pediatric intracranial neoplasms (1). Medulloblastoma, like all the other types of cancer, results from alterations in the equilibrium between cell growth and cell death, which drives the proliferation of cerebellar granule neuronal precursors. This equilibrium is under the control of different metabolic pathways. The most important ones for medulloblastoma-related carcinogenesis include wingless-type MMTV integration site family (WNT)/ CTNNB1 and AKT1 Kinase/ NFKB inhibitor interacting Ras-like 1 (AKT/NF-κB) (2). The WNT/β-catenin and AKT/NF-κB pathways include several transcription factors that control the expression of several cancer-related genes. It is noteworthy that the two pathways induce the expression of three important oncoproteins, namely neuroblastoma MYC oncogene (N-MYC), myc-proto-oncogene Protein (C-MYC) and cyclin D1 (1, 3). Amplification of *N-MYC* and/or *C-MYC* occurs in 5-10% of medulloblastoma cases (4). The MYC family of proteins acts as transcription regulators that play key roles in cell cycle progression, transformation and angiogenesis (5). Furthermore, amplifications as well as high transcriptional levels of *C-MYC* are associated with an unfavorable survival outcome of medulloblastoma patients (6, 7).

Aggressive multimodal therapy with surgery and adjuvant radio- and chemotherapy results in most cases in serious neuropsychological and behavior handicaps. Most of the anticancer cytotoxic agents, when effective, destroy tumor cells by activating programmed cell death pathways (8, 9). Apoptosis is the most common and well-defined form of programmed cell death, which is triggered in response to different anticancer agents (10, 11). The extent of apoptosis induction has been suggested as a practical predictor of tumor chemosensitivity *in vitro* (12). Apoptosis is a major form of cell death of cancer cells including of medulloblastomas, either spontaneous or induced by radiation and chemotherapeutic agents (13-16). Indeed, it has been previously shown that inhibition of apoptosis pathways led to resistance to apoptosis and unfavorable outcome in medulloblastoma (17-20). In addition to apoptosis, several agents trigger cell death through necrosis, another regulated form of cell death (21). In contrast to apoptosis, necrosis

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triggers an acute inflammatory response (22, 23).

Among the drugs used to treat medulloblastoma patients are vincristine (microtubule interfering agent, which inhibits cell division during early mitosis) and lomustine (DNA-alkylating agent) (24-26). Unfortunately, these drugs have detrimental side-effects, and only little is known about their mode of action (27).

In the present study, we assessed the cytotoxic effects of vincristine and lomustine on primary medulloblastoma cells and investigated the link of their activity with the expression of different cancer-related genes.

Materials and Methods

Pathology. Twelve biopsy tissue samples from consenting medulloblastoma patients were diagnosed according to the morphological criteria of the most recent WHO brain tumor classification (28), and the diagnoses were confirmed by the routine liplet microscopic examination of formalin-fixed and paraffin embedded sections that were stained with hematoxylin and eosin (H&E).

Cell culture. Following neurosurgery, a piece of the freshly resected tumor was minced in few drops of complete media (DMEM:F12 50:50 with supplements) using scalpel blades to obtain fine pieces (less than 1 mm in size), and then further pipetted for maximum cell dispersion. Consequently, the fine pieces of tumor were distributed, as drops, in sterile 60 mm tissue culture plates and then covered with a sterile coverslip. The minced tissue was then covered with additional complete medium, which was changed weekly. Confluent grown cells incubated at 37˚C with 5% CO_2 were split using 0.25% Trypsin/1 mM EDTA and stored in liquid nitrogen for further experiments. These cells grew as adherent monolayers in DMEM:F12 50:50 media with supplements, with various shapes from branched to oval or fibrous-shaped. They differed in their size and revealed large round-to-oval nuclei. Primary cell identity was confirmed prior to use by immunostaining for neuronal cell markers Neurofilament 3 (NF-M), Hu-antigen D (HuD) and nestin. Cells were used at low passages (4-8) (Table I).

Medulloblastoma DAOY cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA USA).

Chemotherapeutic agents. Vincristine sulfate (Sigma, St. Louis, MO, USA) and lomustine were dissolved in dimethyl sulfoxide and used at the indicated concentrations.

Cytotoxicity assay. Cells were seeded into 96-well plates at 0.5-1×10^4 viable cells/well and incubated overnight in complete media at 37˚C and 5% CO_2. The medium was replaced with fresh (100 μl) containing the desired concentrations of the drugs. After 20 h, 10 μl of the WST-1 reagent (Amersham, Piscataway, NJ, USA) was added to each well and the plates were incubated for 4 h at 37˚C. The amount of formazan dye formed was quantified using an ELISA reader at 450 nm absorbance.

Cellular lysate preparation. Treated and non-treated cells were washed with PBS and then scraped in RIPA buffer (150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 50 mM Tris-HCl (pH 7.5)), supplemented with protease inhibitors (Aprotinin 2 μg/ml, Leupeptin 0.5 μg/ml, Pepstatin A 0.7 μg/ml and PMSF 174 μg/ml), (Sigma, St. Louis, MO, USA). Lysates were homogenized and then centrifuged at 14000 r.p.m at 4˚C for 15 min in an Eppendorf microcentrifuge. The supernatant was removed, aliquoted and stored at –80˚C.

Table I. *Characteristics of medulloblastoma primary cells and the age of their corresponding patients. The number of stars indicates the growth rate of cells in culture (*: slow, ***: fast).*

Medulloblastoma samples	Gender	Age/ years	Growth rate	Subtype
MED-1	M	7	***	Desmoplastic
MED-2	M	6	**	Desmoplastic
MED-4	M	28	***	Desmoplastic
MED-5	M	6	***	Classic
MED-6	F	8	***	Extensive nodularity
MED-7	M	2	*	Extensive nodularity
MED-8	M	9	***	Classic
MED-9	F	Y	**	Classic
MED-12	M	6	**	Desmoplastic
MED-13	F	3	**	Desmoplastic
MED-18	M	4	*	Classic
MED-16	F	10	**	Classic
DAOY	M	4	***	Desmoplastic

Immunoblotting. SDS-PAGE was performed using 12% separating minigels. Equal amounts of protein extract (30 μg) from different samples were placed in boiling water for 2 min in the presence of SDS gel sample buffer (0.5M Tris pH 6.8, 10% glycerol, 10% SDS, 5% 2-mercaptoethanol, 1% bromophenol) and electrophoresed for 2 hours at 75V. After transfer onto polyvinylidene difluoride membrane (PVDF), the membrane was incubated overnight with the appropriate antibodies. Visualization of the second antibody was performed using a chemiluminescence detection procedure according to the manufactures protocol (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The antibodies were directed against p21 (F-5), p16 (50.1), p53 (DO-1), pRB (IF8), CTNNB1 (9F2), TRKC (H-130), NF-M (1A2), HuD (E-1), Nestin (10C2), β-actin (C-11) and α-Tubulin (TU-02) from Santa Cruz, and C-Myc (9E10) and STK15 (4) from BD Biosciences (San Jose, CA, USA).

Annexin V and flow cytometry. Sub-confluent cells were either sham-treated or challenged with vincristine (0.2 μM), lomustine (168 μM) and the combination of these drugs, whereupon cells were incubated in DMEM/F12 media with supplements. Detached and adherent cells were harvested 72 hours later, centrifuged and re-suspended in 1 ml PBS. Cells were then stained by propidium iodide (PI) and Alexa Fluor 488 annexin V, using Vibrant Apoptosis Assay kit #2 (Molecular probes, Carlsbad, CA USA). Stained cells were analyzed by flow cytometry. The percentage of cells stained was determined by the FACScalibur apparatus and the Cell Quest Pro software from Becton Dickinson (San Jose, CA, USA).

Cell cycle analysis by flow cytometry. Sub-confluent cells were treated with vincristine (0.2 μM) or lomustine (84 μM), and then harvested at various times (0-30 h), centrifuged and re-suspended in 1 ml PBS before being fixed by drop-wise addition of 3 ml of

absolute methanol. Fixed cells were centrifuged, re-suspended in 50 μl of RNase (1 mg/ml) and incubated for 30 min at room temperature, followed by the addition of 1 ml of 0.1 mg/ml of PI. Cells were analyzed for DNA content by flow cytometry (Becton Dickinson, San Jose, CA, USA). The percentage of cells in the different cell cycle phases was determined by using Cell Quest software (Becton Dickinson)

RNA extraction, cDNA synthesis and RT-PCR assay. Total RNA was extracted from untreated cells using Tri® Reagent (Sigma) and the yield was quantitated spectrophotometrically. The integrity of total RNA was assessed on 2% agarose gel. Following the manufacturer's instructions, single stranded cDNA was synthesized from 200 ng of total RNA using MMLV Reverse Transcriptase and oligo dT_{18} (Roche, Branchburg, NJ, USA). The cDNA was amplified for 30 cycles under the following conditions: melting temperature (95˚C) for 50 s, annealing temperature (See table II) for 50 s, and extension temperature (72˚C) for 1 min. The PCR reactions were repeated two times. The PCR products were separated by electrophoresis on a 2% agarose gel at 80 V for an hour. The sequences of the primers used were as follows: *C-MYC*: forward GGAAGAAATTCGAGCTGC; reverse GCTGTCGTTGAGAGGGTA; *STK15*: forward GCAGATTTTGGGTGGTCA; reverse TTCTCTGAGCATTGGCCT; *CDKN1A*: forward ATGAAATTCACCCCCTTTC; reverse CCCTA GGCTGTGCTCACTTC; TRKC: forward AGCACTGCATCGAGTT TGTG; reverse AGTGGGTTTTTGGCAATGAG; *CDKN2A*: forward CCCAACGCACCGAATAGTTA; reverse ACCAGCGTGTCCAGG AAG; *TP53*: forward CAGTCTACCTCCCGCCATA; reverse CCACAACAAAACACCAGTG; *RB*: forward TCCCATGGATTC TGAATGTG; reverse AGAGCCATGCAAGGGATTC; *CTNNB1*: forward AGCCTTCCTTCCTGGGCATG; reverse CTACTGC CTCCGGTCTTCC; *ACTB*: forward CCCAGCACAATGAAGA TCAAGATCAT; reverse ATCTGCTGGAAGGTGGACAGCGA

Quantification of protein and RNA expression levels. The expression levels of RNAs and proteins from both treated and non-treated cells were measured using the densitometer (BIO-RAD GS-800 Calibrated Densitometer, Hercules, CA, USA). Films were scanned and the protein signal intensity of each band was determined. Dividing the obtained value of each band by the values of the corresponding internal control allowed a correction of the loading differences. The fold of induction was determined by dividing the corrected values that corresponded to the treated samples by that of the non-treated one (time 0).

Statistical analysis. The Fisher's exact test was used to evaluate the significance of the differentially expressed proteins on apoptosis induced by different agents. A *p*-value less than 0.05 was taken as the level of significance.

Results

Medulloblastoma cells are resistant to the cytotoxic effect of vincristine and lomustine. To investigate the cytotoxic effect of vincristine and lomustine on medulloblastomas, we made use of low passage primary cell cultures and the WST-1 assay. Cells were seeded into microtiter plates and treated with increasing concentrations of these drugs for 24 hours, and then the cytotoxic effect was measured.

In response to vincristine, primary medulloblastoma cells and the DAOY cell line exhibited clear resistance throughout increasing concentrations of the drug up to 0.4 μM. MED-9 cells showed the highest sensitivity among medulloblastoma primary cells, with a median lethal concentration (LC_{50}) of 0.07 μM (Figure 1).

Treating these cells with higher concentrations of vincristine did not significantly increase the cytotoxic effect. MED-12 and MED-18 cells exhibited somewhat higher sensitivity, with an LC_{50} of 2 μM (Figure 1A). At the same concentration the survival of the other medulloblastoma cells ranged from 60% to 90% (Figure 1).

By contrast, treating the same cells with different concentrations of lomustine showed dose-dependent increase in their cytotoxicity and differential response (Figure 1B). The LC_{50} ranged from 84 μM in MED-9 and MED-2 cells to 265 μM in DAOY cells, which were the most resistant. MED-9 and MED-12 were the most sensitive cells to lomustine. These results indicate that medulloblastoma cells are highly resistant to vincristine and exhibit mild and differential sensitivity to lomustine.

Apoptotic response of medulloblastoma cells to vincristine, lomustine and their combination. In order to identify the type of cell death triggered by vincristine and lomustine, sub-confluent (~60%) medulloblastoma primary cells along with DAOY cell line were treated with vincristine (0.2 μM), lomustine (84 μM and 168 μM) or the combination (vincristine 0.2 μM plus lomustine 84 μM) for three days. Cells were harvested, stained with annexin V and propidium iodide (PI), and then sorted by flow cytometry. This allowed the discrimination of intact living cells (V^-/PI^-), early apoptotic (V^+/PI^-), late apoptotic (V^+/PI^+) and necrotic (V^-/PI^+).

Figure 2A shows raw data for three cell cultures and indicates that both drugs mainly triggered apoptosis. To simplify these results, apoptosis was taken as the sum of early and late apoptosis, subtracted from the sum of early and late apoptosis of the corresponding untreated control sample, and then presented as histogram charts (Figure 2B-E). Since histological studies revealed a high proportion of apoptotic bodies in medulloblastoma samples, we also assessed the extent of spontaneous apoptosis in primary medulloblastoma cells. This proportion ranged from (8.23% to 20%), with an average of about 10%. However, MED-6 cells were revealed to undergo very high proportion of spontaneous apoptosis (40%).

Challenging these cells with vincristine or lomustine showed clear variation in their response (Figure 2C, D). In the case of vincristine, apoptosis ranged between 7.6% in MED-12 cells to 56.7 in MED-5 cells. However, in the case of lomustine treatment, apoptosis ranged between 8.6% in MED-7 and MED-13 cells to 57.3% in MED-12 cells. The

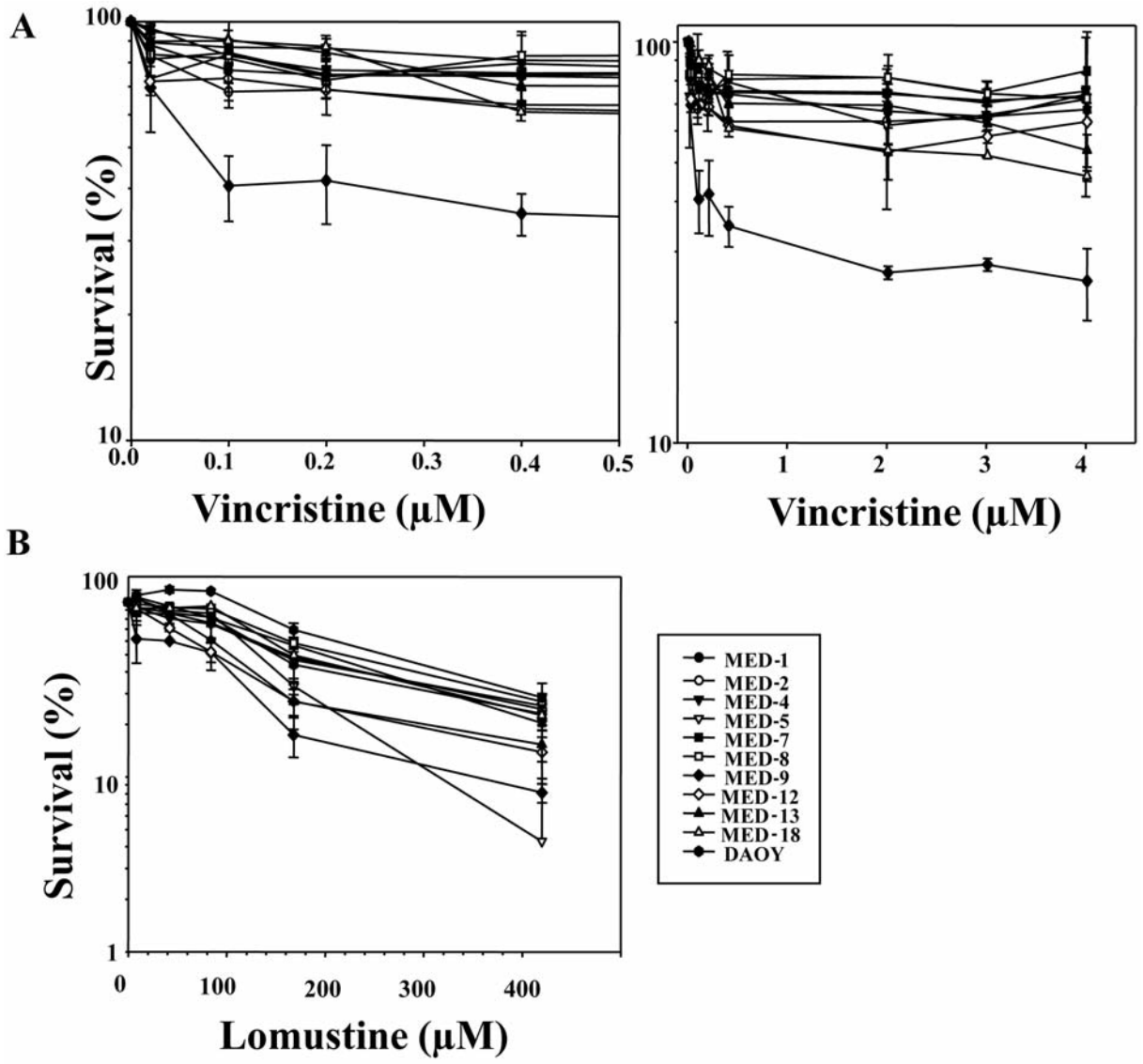

Figure 1. *Cytotoxic effect of vincristine and lomustine on medulloblastoma cells. Cells were seeded in microtiter plates, then either mock-treated or challenged with increasing concentrations of vincristine (A) and lomustine (B). The cytotoxic effect of these drugs was determined using the WST-1 assay. Error bars represent standard deviations of three different experiments.*

difference between the most sensitive and the most resistant responses were calculated to be 7.5- and 6.7-fold, respectively. In order to discriminate these cells according to their response to both drugs, the average of their apoptotic percentages were determined: 19.71% for vincristine treatment and 24.8% for lomustine treatment. Cells that showed a percentage of apoptosis higher than the corresponding average were considered sensitive, while those that showed a percentage of apoptosis nearly equal to the average level (taking the error bars into consideration) were considered mildly responsive. However, cells with a percentage of apoptosis lower than the average value were considered resistant. Thereby, medulloblastoma cells MED-4, -5, -6, -13, -18 and DAOY cells were considered to be sensitive to vincristine. The percentages of apoptosis for these cells were 35.3%, 56.7%, 27.62%, 28.9%, 34.7% and 66.04%, respectively. MED-1 and -8 cells exhibited a mild response; their apoptotic percentage did not exceed 22%. On the other hand, MED-2, -7, -9, -12 and -16 remained very resistant, exhibiting a low percentage of apoptosis ranging from 7.57% to 12.63% (Figure 2C).

Cells were then treated with two different concentrations of lomustine (84 μM and 168 μM). In response to the low concentration, only few primary cells reached 30%

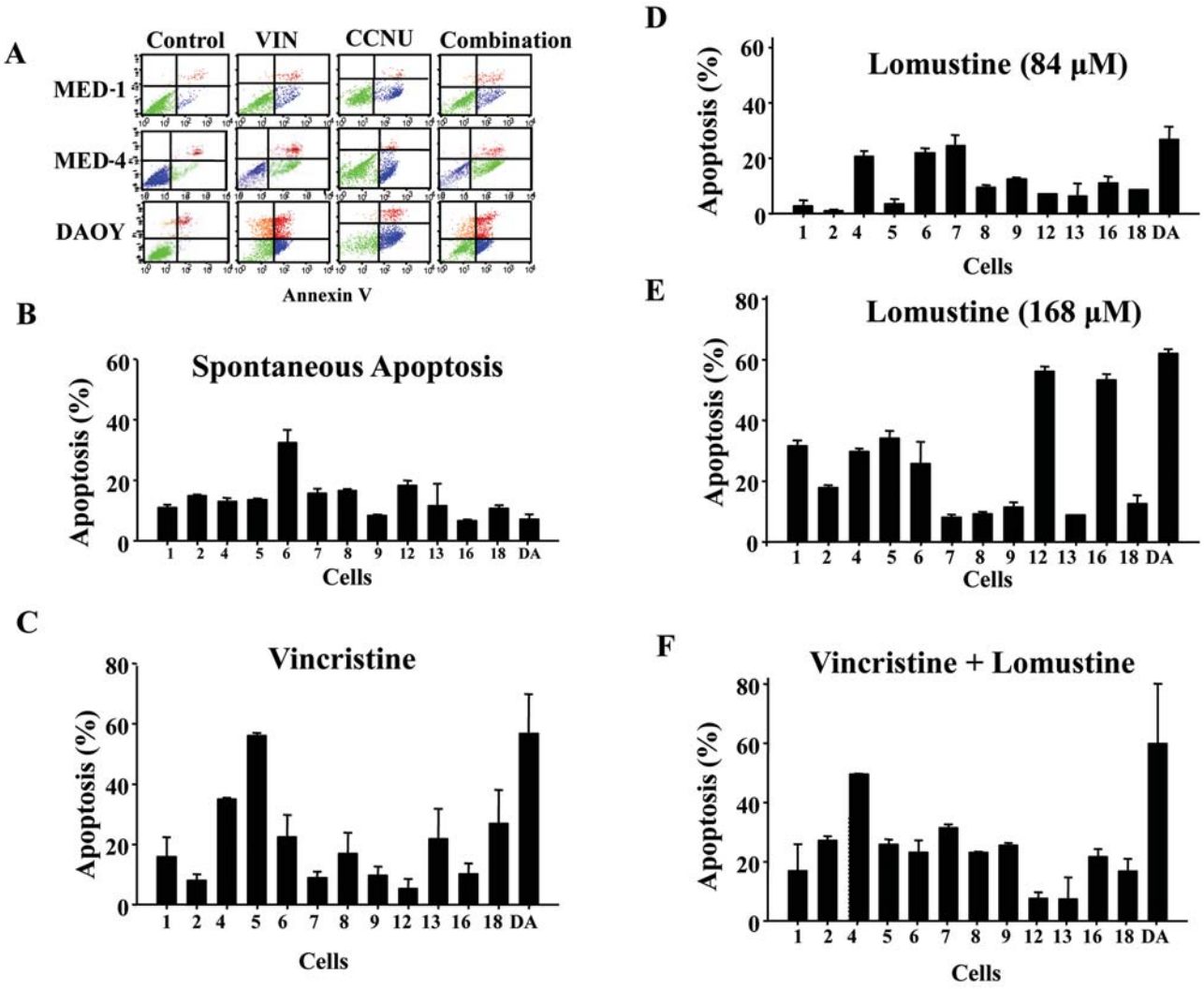

Figure 2. *Apoptotic response of medulloblastoma cells to vincristine and lomustine. Cells were either sham-treated or challenged with vincristine (0.2 μM), lomustine (84 μM and 168 μM), or a combination of vincristine (0.2 μM) and lomustine (84 μM) for 72 hours and then the percentage of cell death was measured by annexin V/flow cytometry (A). B- E: Histograms showing the proportion of apoptotic cells as indicated. Error bars represent standard deviations of three different experiments. The numbers under the X axis correspond to the suffix of medulloblastoma primary cell designations. DA: DOAY.*

apoptosis, while others were highly resistant (Figure 2D). Treatment with the higher concentration of lomustine divided these cells into three groups; the first group contained cells with high sensitivity: MED-1, -4, -5, -6, -12, -16 and DOAY cells. These cells exhibited high percentages of apoptosis: 32.8%, 30.38%, 35.75%, 30.76%, 57.25%, 54.67% and 62.98%, respectively. The second group consisted of only MED-2, which showed a mild response, with a proportion of apoptosis of 18.4%. The third group included MED-7, -8, -9, -13 and -18, which showed high resistance to lomustine treatment. The proportions of apoptosis in these cells were 8.62%, 9.59%, 12.43%, 8.67% and 14.47%, respectively. Notably, 60% of medulloblastoma cells showed high resistance to this drug (Figure 2D).

Since vincristine and lomustine are used in combination in many anti-medulloblastoma regimens, the effect of the combination of these two agents on medulloblastoma primary cells was investigated. Figure 2E shows that medulloblastoma cells had differential response to this combination. Indeed, the effect was either epistatic (MED-1, -6, -12, -16 and DAOY), additive (MED-3, -4, -7, and -8), synergistic (MED-2 and -9) or antagonistic (MED-5, -13 and -18) (Table II).

Effect of vincristine and lomustine on the cell cycle of medulloblastoma cells. To examine the effect of vincristine and lomustine on the cell cycle progression of medulloblastoma cells, sub-confluent (~60%) cells were

Table II. *Effect of vincristine, lomustine and their combination on medulloblastoma primary cells and DAOY cell line. The numbers indicate the percentages of induced apoptosis. Sum: Add up of lomustine + vincristine percentages.*

Samples	Vincristine	Lomustine	Sum	Combination	Effect
MED-1	15.76	2.68	18.44	16.88	Epistatic
MED-2	8.02	0.93	8.95	23.11	Synergistic
MED-4	34.89	20.62	55.51	49.49	Additive
MED-5	55.93	3.57	59.5	24.3	Antagonistic
MED-6	27.62	23.04	50.66	26.04	Epistatic
MED-7	8.93	24.48	33.41	31.39	Additive
MED-8	16.93	9.42	26.35	21.7	Additive
MED-9	9.69	12.44	22.13	25.51	Synergistic
MED-12	5.23	6.8	12.03	7.59	Epistatic
MED-13	21.78	6.21	27.99	7.32	Antagonistic
MED-16	12.63	12.7	25.33	23.55	Epistatic
MED-18	26.84	8.57	35.41	16.8	Antagonistic
DAOY	56.77	26.68	83.45	59.88	Epistatic

treated with vincristine 0.2 μM or lomustine 84 μM for different time intervals (0-30 h). Subsequently, cells were stained with propidium iodide and DNA content was analyzed by flow cytometry.

Figure 3A shows the effect of vincristine and lomustine on the cell cycle of MED-1 cells. At time zero (0h) most of the cells (64.02%) were at the G_0/G_1 phase with 2N DNA content. After 8 hours, a significant increase in the proportion of cells at the G_2/M phase had occurred in control cells, as well as in cells treated with vincrstine or lomustine, reaching up to 32.5%. This clearly indicates that these drugs do not have any effect on these cells at the G_0/G_1 phase of the cell cycle (Figure 3B). However, after 16 hours of treatment, while the proportion of G_2/M cells decreased to 13.1% in the sham-treated cells, it remained at 33.7% and 29.5% in the vincristine- and lomustine- treated cells, respectively (Figure 3B). Interestingly, this proportion of cells at G_2/M was maintained for the next 24 hours (Figure 3B). This clearly shows that the two drugs induced cell cycle delay at the G_2/M phase of the cell cycle in MED-1 cells.

Figure 3C shows the effect of vincristine and lomustine on MED-2 cells. These cells exhibited clear cell cycle delay at the G_2/M phase following treatment with vincristine. However, no significant delay was observed in response to lomustine, indicating that these cells are defective in lomustine-dependent cell cycle arrest at the G_2/M phase. Moreover, MED-18 cells showed no response to either drug, proving a defect in both vincristine- and lomustine-dependent cell cycle arrest at the G_2/M phase (Figure 3D).

Therefore, in response to vincristine, 4 out of 10 of the medulloblastoma cells (MED-1, -2, -4 and -12) showed cell cycle delay at the G_2/M phase, while 6 out of 10 of these cells (MED-5, -7, -8, -9, -13 and -18) were defective for such delay. However, only 20% of medulloblastoma cells (MED-1 and MED-5) exhibited G_2/M cell cycle delay following lomustine treatment. Based on these results, medulloblastoma primary cells can be divided into four groups: cells that were arrested at the G_2/M phase by both drugs (MED-1), cells that were arrested at the G_2/M phase either by vincristine or by lomustine: MED-2 and -5, and cells that were not delayed by vincristine or by lomustine (MED-7, -8, -9, -13 and -18). As a result, 50% of medulloblastoma primary cells were defective in G_2/M cell cycle arrest in response to vincristine and lomustine treatment.

p16 is up-regulated in response to vincristine and lomustine in medulloblastoma cells. p16 is a cycline-dependent kinase inhibitor that plays a key role in cell cycle control. It has been found to be mutated or transcriptionally inhibited in different neoplasma, including brain and skin cancer (29). To study the effect of vincristine and lomustine on the expression of p16 protein, medulloblastoma MED-5 cells were treated with 0.2 μM of vincristine or 84 μM of lomustine. Cells were then incubated for different periods of time and whole-cell extracts were prepared and used for Western blot analysis using the appropriate antibodies, and β-actin was used as internal control. The increase in the level of p16 protein was considered as induction only when the protein level following treatment was twice that in the control sample. Figure 4 shows that p16 protein was up-regulated in response to both drugs. In response to vincristine, p16 was accumulated reaching a level 2.3-fold higher after 6 hours of treatment, then it decreased. For lomustine, the treatment caused a dose-dependent up-regulation of p16 for up to 16 hours of treatment, reaching a level 10-fold higher than the basal level, which then decreased sharply and was not detectable after 24 hours of treatment (Figure 4). Similar results were obtained in the MED-4 cell culture. This shows that the cyclin-dependent kinase inhibitor p16 is up-regulated upon cellular exposure to lomustine and vincristine, the effect of the DNA damaging agent (lomustine) was 5 times more effective than vincristine.

Expression profile of cancer genes in medulloblastoma cells. Cancers are genetically heterogeneous, and tumor progression is characterized by step-wise accumulation of genetic and epigenetic abnormalities, in addition to variation in the expression of a wide range of genes (30). Therefore, we sought to investigate the molecular basis of the differential response of medulloblastoma cells to vincristine and lomustine. To this end, we studied the mRNA and protein expression levels of 8 cancer-related genes, in the different medulloblastoma cells. Whole cell extracts and total RNA were prepared and their levels were assessed by immunoblotting using specific antibodies and RT-PCR, respectively.

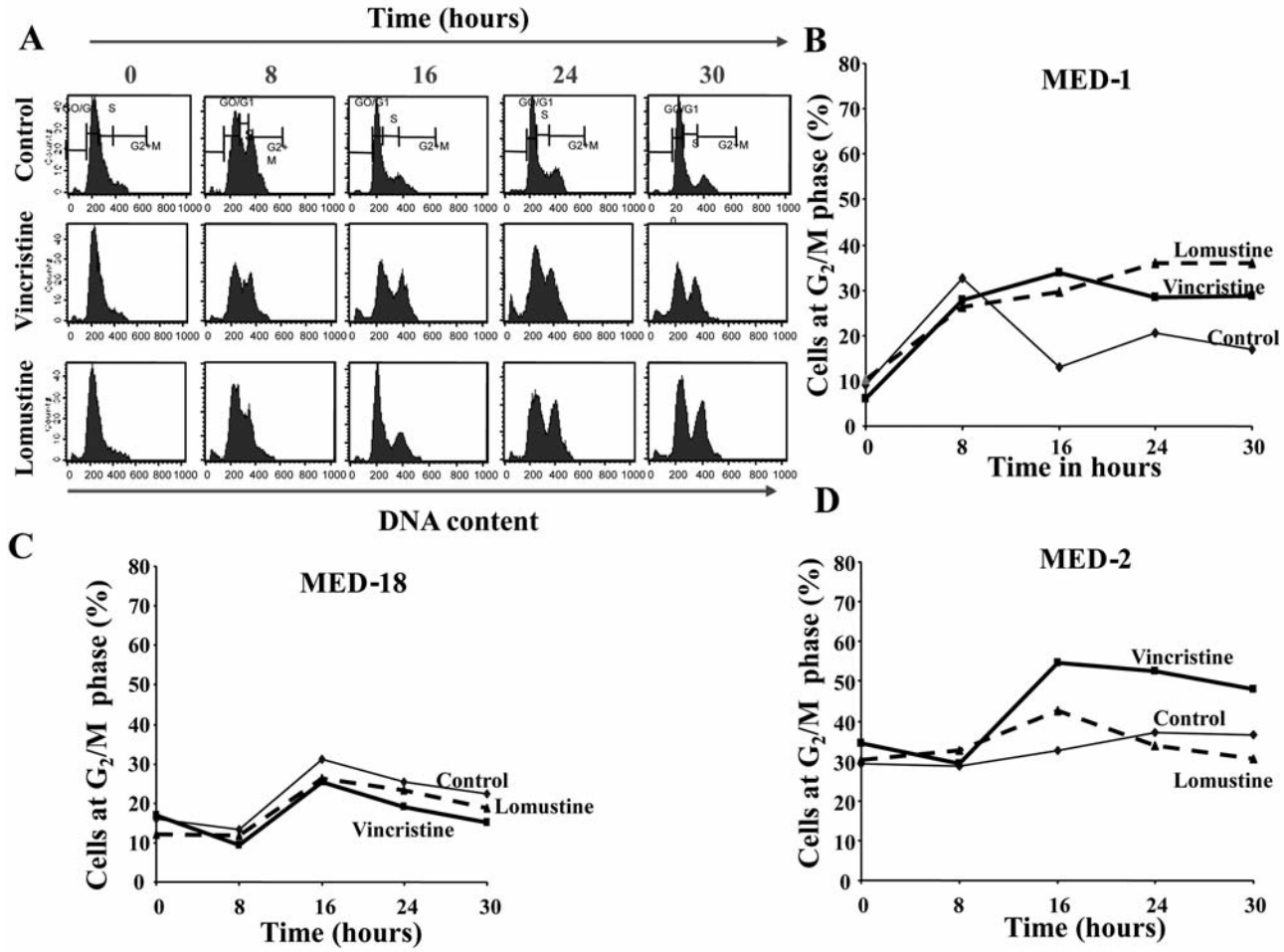

Figure 3. *Effect of vincristine and lomustine on the cell cycle of medulloblastoma cells. Cells were either sham-treated (Control) or challenged with vincristine (0.2 μM) or lomustine (84 μM) for the indicated periods of time. Subsequently, cell cycle status was analyzed by flow cytometry. A: FACScan analysis. B-D: Graphs illustrating the proportion of cells at G_2/M phase.*

Figure 5 shows the RT-PCR products of the 8 genes and their corresponding protein levels. Both the median normalized mRNA level (MNPL) and the median normalized mRNA level (MNML) were defined for each of these 8 genes. Proteins and mRNAs with expression levels higher than the corresponding MNPL or MNML were considered to be highly expressed. However, those that showed expression levels lower than the corresponding MNPL or MNML were defined as lowly expressed. Genes with levels that were equal or nearly equal to the corresponding MNPL or MNML were considered "normally" expressed. Figure 5 shows the great variation in the mRNA and protein expression levels of the 8 genes examined in the various medulloblastoma cells. Interestingly, for most of the genes and cell cultures, the mRNA levels did not reflect the corresponding protein levels. This is well exemplified in MED-5 and MED-12 cells, where the mRNA levels of various genes were normally or highly expressed, while the corresponding proteins were almost undetectable. The best examples are *CDKN2A* and *C-MYC* (Figure 5). For *C-MYC*, the MNML was 0.84 while the MNPL was 0.16. Accordingly, the mRNA of this gene was highly expressed in MED-4, -5, -7, -12 and -13 (Table III), while the protein was highly expressed in 5 other medulloblastoma cells (MED-1, -2, -6, -9, and -18) (Table IV). For the *CDKN2A* gene, the MNML level was 0.93 and the MNPL level was 0.32. Accordingly, the highest mRNA expression was observed in MED-2, -5, -7, -9, -12 and -13, while p16 protein was highly expressed in MED-6, -7, -9 and -16. For this gene, only MED-2 and MED-9 cells showed high expressions of both mRNA and protein (Tables III and IV).

Concerning the expression of the *STK15* and *TRKC* genes, both the mRNA and the protein levels were highly heterogeneous (Figure 5). For *STK15* the MNML was 0.45 and the MNPL level was 0.2. The mRNA expression for this

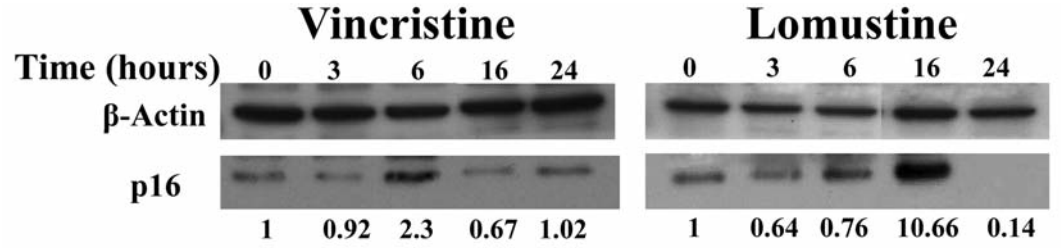

Figure 4. *Effect of vincristine and lomustine on the expression of p16. MED-5 cells were either sham-treated or challenged with vincristine (0.2 μM) and lomustine (84 μM) for the indicated periods of time. Whole-cell extracts were prepared and 30 μg of proteins were used for immunoblotting assay, using the indicated antibodies. The numbers under the bands indicate the fold induction, taking time 0 as control.*

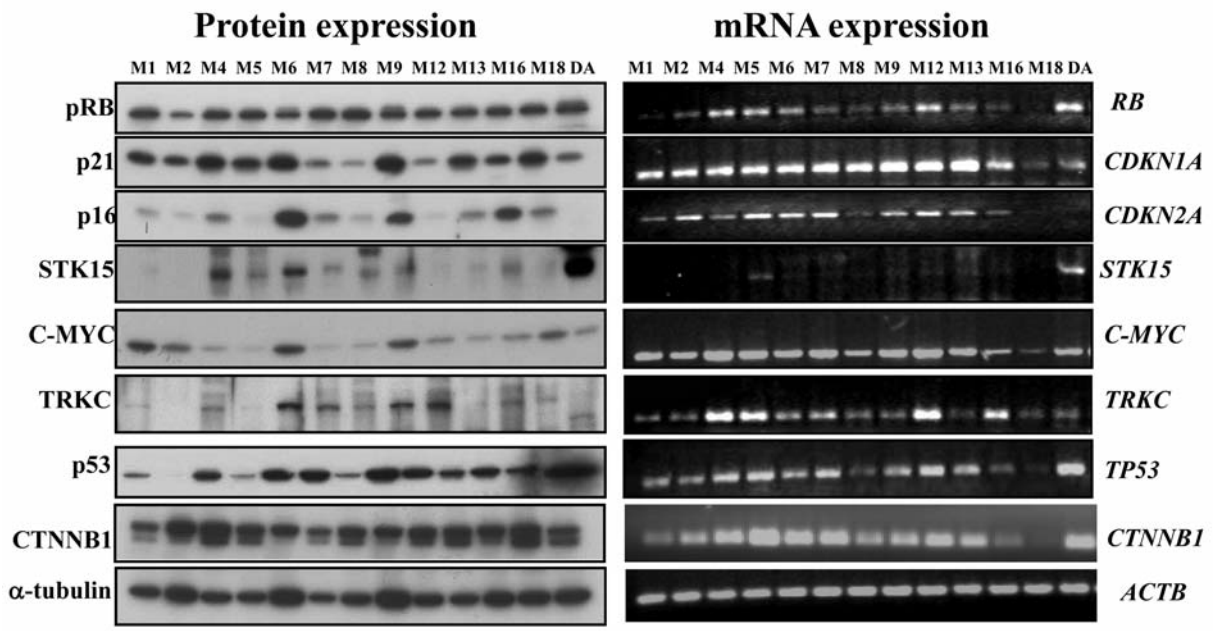

Figure 5. *Protein and mRNA expression profiles for medulloblastoma cells. Left panel: Whole-cell extracts were prepared from medulloblastoma cells and utilized for immunoblotting assay. Specific antibodies as indicated were used; GAPDH was used as internal control. Right panel: Total RNA was extracted from these cells, and RT-PCR was performed for the indicated genes. β-Actin (ACTB) was used as internal control. M: Medulloblastoma cells.*

gene was at its highest level in MED-5 cells (0.56), and at its lowest level in MED-1 cells (0.31). The *STK15* mRNA was undetectable or exhibited very low levels in several MED cells. By contrast, the STK15 protein was highly expressed in MED-4, -5, -6, -8 and -9 (Tables III and IV). Interestingly, while expression of *STK15* mRNA was very low in MED-4 and MED-6, its corresponding protein was highly expressed in these cells (Figure 5). The MNML of the *TRKC* gene was 0.6, while the MNPL level was 0.08. In fact, most medulloblastoma cells expressed very low level of TRKC protein. In both cases, the difference between the highest level and the lowest one was 23-fold. For *TRKC*, the mRNA levels of several genes were normal or high; however, the corresponding proteins were almost undetectable (Figure 5).

For the RB and *CTNNB1* genes, the mRNA levels were highly heterogeneous, with the difference between the highest levels and the lowest ones being 23- and 16- fold, respectively. However, the protein levels were similar, with the difference between the highest and the lowest values being only 2.8-fold for *RB* and 2.69-fold for *CTNNB1*. CTNNB1 protein was found to be highly expended in MED-4, -5, -13 and -18 cells while the pRB protein level was high in MED-1, -4, -5, -7 and -8 (Figure 5 and tables III and IV).

Regarding the *TP53* gene, the MNML level was 1.1. MED-5 showed the highest expression level (1.9), while MED-18 showed the lowest level (0.1), and the fold-difference between them was 19. Accordingly, *TP53* mRNA was highly expressed in MED-4, -5, -9 and -12 cells (Table III). Moreover, the p53

Table III. *The apoptotic response of medulloblastoma cells to vincristine and lomustine and the mRNA expression levels for the indicated genes. The numbers indicate the percentages of induced apoptosis. The bolded and underlined numbers were used to highlight the correlation between the proportions of lumostine-induced apoptosis and the expression levels of TRKC mRNA.*

Medulloblastoma samples	Vincristine (0.2 μM) Apoptosis average: **19.7**	Lomustine (168 μM) Apoptosis average: **24.8**	Combination Apoptosis average: **23**	mRNA expression							
				P53 MNML **1.1**	CDKN1A MNML **1.4**	CDKN1A MNML **0.93**	PRB MNML **0.5**	C-MYC MNML: **0.84**	STK15 MNML **0.45**	TRKC MNML **0.6**	CTNNB1 MNML **0.44**
MED-1	15.76	31.49	16.88	0.77	1.2	0.73	0.03	0.71	0.31	0.45	0.21
MED-2	8.015	17.76	27.2	0.86	1.2	1.3	0.46	0.55	0.32	0.04	0.4
MED-4	34.9	**29.65**	49.49	1.5	1.3	0.82	0.66	1.38	0.38	**0.93**	0.45
MED-5	55.93	**34.01**	25.82	1.9	1.4	1.44	0.69	1.2	0.56	**0.92**	1.48
MED-6	22.4	25.6	23.07	0.97	1.4	0.96	0.68	0.86	0.43	0.43	0.48
MED-7	8.93	7.94	31.4	1.2	1.3	1.3	0.56	1.1	0.45	0.57	0.6
MED-8	16.925	9.03	23.1	0.52	1.3	0.33	0.44	0.46	0.44	0.36	0.22
MED-9	9.685	11.23	25.5	1.4	1.4	1.3	0.55	0.92	0.43	0.4	0.33
MED-12	5.23	**56.06**	7.59	1.7	1.8	1.4	0.64	1.42	0.44	**0.89**	0.46
MED-13	21.8	8.67	7.32	1.2	2.1	1.2	0.51	1.05	0.44	0.36	0.46
MED-16	10.13	**53.24**	21.7	0.48	1.1	0.3	0.50	0.35	0.41	**0.63**	0.18
MED-18	26.84	12.53	16.795	0.1	0.71	0.06	0.04	0.21	0.32	0.44	0.0
DAOY	56.77	62.02	59.88	4.00	0.96	0.041	0.78	0.72	0.95	0.45	1.3

MNML: Median normalized mRNA level.

Table IV. *The apoptotic response of medulloblastoma cells to vincristine and lomustine and the protein expression levels for the indicated genes. The numbers indicate the percentages of induced apoptosis. The bolded and underlined numbers were used to highlight the correlation between the proportions of vincristine- and combination-induced apoptosis and the expression levels of STK15 and CTNNB1 proteins, respectively.*

Medulloblastoma samples	Vincristine (0.2 μM) Apoptosis average: **19.7**	Lomustine (168 μM) Apoptosis average: **24.8**	Combination Apoptosis average: **23**	Protein expression							
				p53 MNPL **0.45**	p21 MNPL **0.7**	p16 MNPL **0.32**	pRB MNPL **0.61**	C-MYC MNPL **0.16**	STK15 MNPL **0.2**	TRKC MNPL **0.08**	CTNNB1 MNPL **1.2**
MED-1	15.76	31.49	16.88	0.13	0.94	0.18	0.84	0.28	0.0	0.0	1.01
MED-2	8.015	17.76	27.2	0.0	0.56	0.10	0.33	0.21	0.0	0.0	1.12
MED-4	**34.9**	29.65	**49.49**	0.62	1.15	0.35	0.77	0.071	**0.5**	0.09	**1.7**
MED-5	**55.93**	34.01	**25.82**	0.29	0.97	0.13	0.73	0.048	**0.32**	0.0	**1.4**
MED-6	22.4	25.6	**23.07**	0.51	0.85	0.75	0.40	0.23	**0.47**	0.20	0.75
MED-7	8.93	7.94	31.4	0.61	0.50	0.43	0.91	0.083	0.08	0.12	0.63
MED-8	16.93	9.03	**23.1**	0.31	0.25	0.17	0.76	0.068	**0.24**	0.07	1.15
MED-9	9.685	11.23	**25.5**	0.61	0.92	0.46	0.42	0.20	**0.23**	0.13	0.78
MED-12	5.23	56.06	7.59	0.58	0.28	0.09	0.56	0.15	0.04	0.23	1.2
MED-13	**21.8**	8.67	7.32	0.65	0.87	0.32	0.62	0.084	0.08	0.0	**1.4**
MED-16	10.13	53.24	21.7	0.57	0.45	0.56	0.48	0.14	0.2	0.02	1.06
MED-18	**26.84**	12.53	16.795	0.46	0.69	0.34	0.53	0.29	0.12	0.04	**1.34**
DAOY	56.77	62.02	59.88	3.8	0.28	0.06	0.63	0.17	1.12	0.003	1.09

MNPL: Median normalized mRNA level.

MNPL level was 0.45, with the fold-difference of 65 between the highest expression level observed in MED-13 cells (0.65) and the lowest expression level detected in MED-2 cells (0.01). Seven out of 12 primary cells showed high expression of this protein: MED-4, -6, -7, -9, -12, -13 and -16 (Table IV). The p53 downstream effector p21 exhibited similar variation in mRNA and protein levels (Figure 5).

Interestingly, similar results were obtained when the mRNA levels of two tumor suppressor genes *CDKN1A* and *TP53* and two oncogenes *C-MYC* and *STK15* were assessed from the tissues of two medulloblastoma samples, MED-13 and MED-18. The expression levels of the four genes were similar to that of the cultured cell samples. Indeed, the four genes were highly expressed in MED-13 as compared to

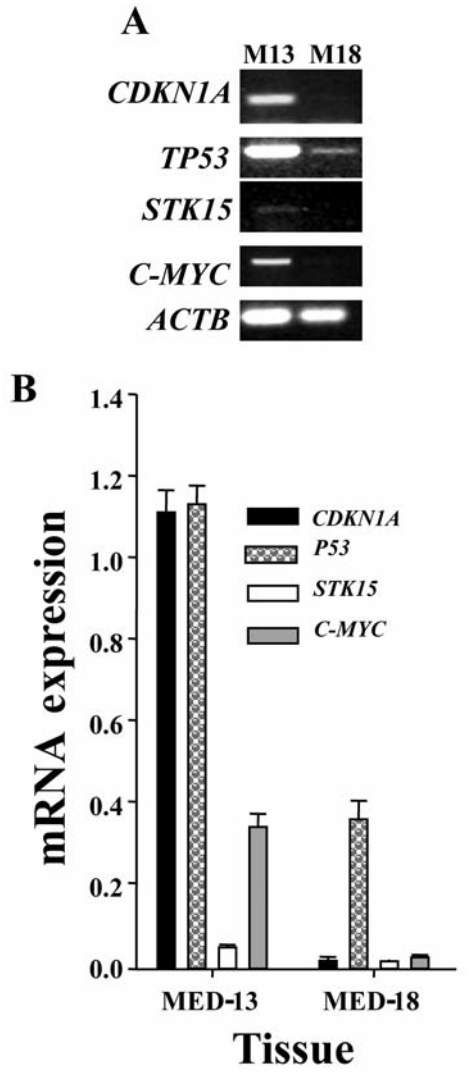

Figure 6. *mRNA expression from medulloblastoma tissue samples. Total RNA was extracted from tissue samples, and the cDNA was synthesized and used to evaluate the mRNA expression for the indicated genes using the RT-PCR technique. β-Actin (ACTB) was used as internal control. A: Ethidium bromide stained agarose gels showing the RT-PCR products. B: Histogram showing the expression levels of the indicated genes. M: Medulloblastoma cells. Error bars represent standard deviations of three different experiments.*

MED-18 in both cell cultures and the corresponding tissues. This shows that the use of low passage primary cells reflects to high extent the situation in the original tumors (Figure 6).

Direct association between the expression levels of CTNNB1/STK15/TRKC and the sensitivity of medulloblastoma primary cells to vincristine and lomustine. Next, we investigated the possible link between the apoptotic response of medulloblastoma primary cells to vincristine and lomustine and the expression levels of the 8 different genes. Table IV shows the existence of a direct correlation between the level of the CTNNB1 protein and the apoptotic response to vincristine treatment. Indeed, all cells that expressed high levels of CTNNB1 (a level higher than the MNPL) showed high response to vincristine; whilst low levels of this protein (lower than the MNPL) correlated with a low proportion of apoptotic cells (Table IV). This indicates the existence of a significant correlation between the expression of CTNNB1 and the response of medulloblastoma cells to vincristine, with a *p*-value of 0.04.

Furthermore, we have shown that the response of medulloblastoma cells to the combination of lomustine and vincristine was also directly related to the expression level of the STK15 protein. Indeed, a high level of this protein kinase correlated with significant apoptosis in all cells, while in 72% of cases, low STK15 levels correlated with resistance to the combination of vincristine and lomustine. Therefore, this correlation was highly significant with a *p*-value of 0.03 (Table IV). In addition, at the mRNA level, *TRKC* expression showed direct correlation with the killing effect of lomustine. Indeed, cells expressing high levels of *TRKC* exhibited high levels of apoptosis in response to lomustine treatment. This indicates a significant link between the mRNA level of *TRKC* and the apoptotic response of these tumor cells to lomustine, with a *p*-value of 0.03 (Table III). However, no correlation was found between the TRKC protein level and the apoptotic response to lomustine (Table III).

Discussion

Vincristine and lomustine are used for the treatment of different neoplasms including medulloblastomas (24-27). To further characterize the action of these agents, we studied their effects on 12 primary medulloblastoma cells and the widely used DAOY cell line. The features of these primary low passage cells may mirror better the characteristics of the original tumors, and thereby could be utilized to carry out experimental studies on molecular pathogenesis and development of more efficient modes of treatment. Indeed, Figures 5 and 6 show that the expression of different genes is similar in primary cells and their corresponding tissues. Remarkably, the ability to form colonies from the isolated cells that derived from the different tissues and then the proliferation rates during the early passages were variable (Table I). Furthermore, immediately after the formation of cell colonies, these cells showed various proportions of spontaneous cell death (floating cells) (Figure 2B). These early phenotypic differences might reflect variations in the tumors before plating. Together, these molecular and cellular characteristics suggest that these medulloblastoma cultures are representative of the tumors of origin.

We have first shown that whilst lomustine exhibited a dose-dependent cytotoxic effect, vincristine cytotoxicity did not increase in a dose-dependent manner, but rather was lost and all cells became resistant while the drug concentrations were still low (Figure 1). This biphasic cytotoxic effect is not due to the presence of two different populations (one resistant and one sensitive), since cells were treated while more than 80% of them were at the G_0/G_1 phase (data not shown). However, this could be owing to the fact that vincristine is a substrate of multidrug-resistance (MDR) proteins such as P-glycoprotein (P-gp) and MDR-associated proteins 1 and 2 (MRP). These proteins are expressed in different cell types and normally protect them against the harmful effects of different xenobiotics, notably vincristine, by extruding drugs out of cells, thus decreasing intracellular drug concentrations (31, 32). It has been previously shown, using mice lacking Mrp1 and P-gp, that these proteins cooperate in protecting the organism from the cytotoxic effect of vincristine (33). Interestingly, it has been recently reported that vincristine treatment increased the expression and activity of the MDR protein transporters in the rat nervous system (34). This suggests that at certain concentration, vincristine induces the MDR proteins, which allows the efflux of the drug and hence leads to cellular resistance.

We then showed that both lomustine and vincristine triggered cell death mainly through the apoptotic pathway in all medulloblastoma cells that were analyzed. However, the response of these cells to these drugs was very heterogeneous, reflecting the large variation in the response of medulloblastoma patients to these drugs (27).

Importantly, when both drugs were combined together, the medulloblastoma cells displayed different responses: epistatic (4 out of 12 primary cells and the DOAY cell line), synergistic (2 out of 12), additive (3 out of 12) and also antagonistic (3 out of 12). Therefore, in 8 out of 13 (61%) cell types, the effect of the combination of both drugs did not enhance the apoptotic response, while in 5 out of 13 (39%), the combination led to more efficient cell killing. Intriguingly, the combination had an antagonistic effect in MED-18, -13 and -5 (Table II). Similar results were obtained when different concentrations of vincristine were used and also when the drugs were added consecutively (data not shown). This differential response may result from great heterogeneity in the genetic make up of these primary cells. Indeed, it is clear from Figure 5 that a great variation in gene expression exists among them. Furthermore, we have shown a clear correlation between the expression of the STK15 protein and the sensitivity of MED cells to the combination of both drugs. It is also possible that medulloblastoma cells express various MDR proteins and at different levels. These proteins are known to confer resistance to various types of cancer (35). In fact, a statistically significant association has been previously found between MDR protein expression and outcome of medulloblastoma patients (36), showing the importance of these proteins in the response of medulloblastomas to chemotherapeutic agents. Therefore, the present results clearly show that the combination of chemotherapeutic drugs does not always lead to better response and that tumor cells may respond differently, which urges tumor-genetics tailored therapy.

In addition to the induction of cell death, we have shown here that vincristine and lomustine inhibit cell proliferation by arresting cells at the G_2/M phase of the cell cycle. Intriguingly, only one cell culture showed this response (MED-1). However, 5 out of 10 cell cultures (50%) were found to be deficient in the G_2/M cell cycle arrest upon treatment with both vincristine and lomustine and 40% of cells showed deficiency in response to one of the drugs. As described for cell death, these results also indicated high heterogeneity in the response of medulloblastoma cells to vincristine and lomustine.

Moreover, we present evidence that lomustine and vincristine up-regulate p16 protein level in medulloblastoma cells. p16 is a key tumor suppressor that blocks the progression of the cell cycle by binding to either CDK4 or CDK6 and inhibiting the action of cyclin D (37-40). The p16 coding gene has been found to be homozygously deleted, mutated or transcriptionally inhibited by methylation in a large number of different human tumor types (29, 38-40). Furthermore, p16 plays key roles in the induction of apoptosis in response to some chemotherapeutic agents such as cisplatin (41). The present results provide the first indication that this tumor suppressor gene is up-regulated in response to various cellular stresses. Therefore, p16 up-regulation following vincristine and lomustine could play great role in the response of medulloblastoma cells to these agents, by inhibiting cell proliferation or triggering cell death.

After showing the great difference in the response of medulloblastoma cells to vincristine and lomustine, we have shown that these cells express various levels of many cancer-related genes that are involved in cell proliferation and cell death. For different genes and in various medulloblastoma cells, a clear discrepancy has been observed between the expression level of their mRNAs and their corresponding proteins. Indeed, while *CDKN2A* mRNA is highly expressed, its corresponding protein is almost undetectable in MED-5 cells. On the other hand, the expression of *STK15* mRNA is very low, while the corresponding protein is highly expressed (Figure 5). This clearly shows changes in the post-transcriptional regulation processes. These include the stability of the mRNA, the implication of miRNAs, translation efficiency, post-translational modifications, as well as protein turnover. All these gene expression regulation processes are under tight control of several proteins/RNA that have recently been shown to play key roles in

carcinogenesis. Since in most cases the level of mRNA does not reflect that of the corresponding protein, and since it is the protein level which is determinant in the function of each gene, it seems more reasonable to link protein expression level, rather than mRNA levels, as markers or predictive tools. In this line of research we have investigated the link between the expression levels of all these genes and the response of medulloblastoma cells to vincristine and lomustine. Interestingly, we have found a great link between the level of the transcription factor CATNNB1 protein and the efficient killing of cells with vincristine. Indeed, all cells that expressed high levels of CATNNB1 were sensitive to the killing effect of vincristine. This explains what has been previously published showing that CATNNB1 status predicts a favorable outcome in childhood medulloblastoma (42). Therefore, the level of the CATNNB1 protein could play an important role as a predictive marker for the treatment of medulloblastoma patients.

Furthermore, we have shown that the sensitivity of medulloblastoma cells to lomustine and vincristine combination was directly related to the expression level of the STK15 protein. Indeed, a high level of this protein kinase correlated well with significant apoptosis in all cells. In line with this result, microarray-based screening for molecular markers in medulloblastoma revealed STK15 as an independent predictor for survival (43). In addition, we also found direct correlation between the mRNA level of *TRKC* and the killing effect of lomustine. Indeed, cells expressing high levels of *TRKC* showed high levels of apoptosis in response to lomustine. In fact, it has been previously shown that the activation of neurotrophin-3 receptor TRKC induces apoptosis in medulloblastomas (44). Furthermore, the expression of TRKC has been found to be linked to a favorable outcome in medulloblastoma (45). Taken together, these results indicate that CATNNB1 and STK15 proteins, as well as *TRKC* mRNA, could constitute important molecular markers for the stratification of medulloblastoma patients.

In summary, the present report shows great variation in the expression of different medulloblastoma-related genes at both the mRNA and protein levels. These variations led to large differential sensitivity to vincristine and lomustine, and also to various responses to the combination of these drugs. Furthermore, we identified *STK15, TRKC* and *CTNNB1* as important genes for predicting the response of medulloblastoma cells to the treatment with these agents.

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References

1 Jozwiak J, Grajkowska W and Wlodarski P: Pathogenesis of medulloblastoma and current treatment outlook. Med Res Rev *6*: 869-890, 2007.
2 Gilbertson RJ: Medulloblastoma: signalling a change in treatment. Lancet Oncol *5*: 209-218, 2004.
3 Kenney AM, Widlund HR and Rowitch DH: Hedgehog and PI-3 kinase signaling converge on Nmyc1 to promote cell cycle progression in cerebellar neuronal precursors. Development *131*: 217-228. Epub 2003 Dec 2003, 2004.
4 Aldosari N, Bigner SH, Burger PC, Becker L, Kepner JL, Friedman HS and McLendon RE: *MYCC* and *MYCN* oncogene amplification in medulloblastoma. A fluorescence in situ hybridization study on paraffin sections from the Children's Oncology Group. Arch Pathol Lab Med *126*: 540-544, 2002.
5 Vita M and Henriksson M: The myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol *16*: 318-330, 2006.
6 Scheurlen WG, Schwabe GC, Joos S, Mollenhauer J, Sorensen N and Kuhl J: Molecular analysis of childhood primitive neuroectodermal tumors defines markers associated with poor outcome. J Clin Oncol *16*: 2478-2485, 1998.
7 Grotzer MA, Hogarty MD, Janss AJ, Liu X, Zhao H, Eggert A, Sutton LN, Rorke LB, Brodeur GM and Phillips PC: MYC messenger RNA expression predicts survival outcome in childhood primitive neuroectodermal tumor/medulloblastoma. Clin Cancer Res *7*: 2425-2433, 2001.
8 Brown JM and Attardi LD: The role of apoptosis in cancer development and treatment response. Nat Rev Cancer *5*: 231-237, 2005.
9 Reed JC: Apoptosis-targeted therapies for cancer. Cancer Cell *3*: 17-22, 2003.
10 Johnstone RW, Ruefli AA and Lowe SW: Apoptosis: a link between cancer genetics and chemotherapy. Cell *108*: 153-164, 2002.
11 Ledgerwood EC and Morison IM: Targeting the apoptosome for cancer therapy. Clin Cancer Res *15*: 420-424, 2009.
12 Wu GS and El-Deiry WS: Apoptotic death of tumor cells correlates with chemosensitivity, independent of p53 or bcl-2. Clin Cancer Res *2*: 623-633, 1996.
13 Fulda S, Scaffidi C, Pietsch T, Krammer PH, Peter ME and Debatin KM: Activation of the CD95 (APO-1/Fas) pathway in drug- and gamma-irradiation-induced apoptosis of brain tumor cells. Cell Death Differ *5*: 884-893, 1998.
14 Soini Y, Paakko P and Lehto VP: Histopathological evaluation of apoptosis in cancer. Am J Pathol *153*: 1041-1053, 1998.
15 Haslam RH, Lamborn KR, Becker LE and Israel MA: Tumor cell apoptosis present at diagnosis may predict treatment outcome for patients with medulloblastoma. J Pediatr Hematol Oncol *20*: 520-527, 1998.
16 Pizem J, Cor A, Zadravec Zaletel L and Popovic M: Prognostic significance of apoptosis in medulloblastoma. Neurosci Lett *381*: 69-73. Epub 2005 Feb 2025, 2005.
17 Hacker S, Dittrich A, Mohr A, Schweitzer T, Rutkowski S, Krauss J, Debatin KM and Fulda S: Histone deacetylase inhibitors cooperate with IFN-gamma to restore caspase-8 expression and overcome TRAIL resistance in cancers with silencing of caspase-8. Oncogene *28*: 3097-3110, 2009.

18 Pingoud-Meier C, Lang D, Janss AJ, Rorke LB, Phillips PC, Shalaby T and Grotzer MA: Loss of caspase-8 protein expression correlates with unfavorable survival outcome in childhood medulloblastoma. Clin Cancer Res *9*: 6401-6409, 2003.
19 Aguilera DG, Das CM, Sinnappah-Kang ND, Joyce C, Taylor PH, Wen S, Hasselblatt M, Paulus W, Fuller G, Wolff JE and Gopalakrishnan V: Reactivation of death receptor 4 (DR4) expression sensitizes medulloblastoma cell lines to TRAIL. J Neurooncol *93*: 303-318, 2009.
20 Li Y, Guessous F, Johnson EB, Eberhart CG, Li XN, Shu Q, Fan S, Lal B, Laterra J, Schiff D and Abounader R: Functional and molecular interactions between the HGF/c-MET pathway and c-MYC in large-cell medulloblastoma. Lab Invest *88*: 98-111, 2008.
21 Zong WX and Thompson CB: Necrotic death as a cell fate. Genes Dev *20*: 1-15, 2006.
22 Jaattela M: Multiple cell death pathways as regulators of tumour initiation and progression. Oncogene *23*: 2746-2756, 2004.
23 Zong WX, Ditsworth D, Bauer DE, Wang ZQ and Thompson CB: Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev *18*: 1272-1282. Epub 2004 May 1214, 2004.
24 Packer RJ, Goldwein J, Nicholson HS, Vezina LG, Allen JC, Ris MD, Muraszko K, Rorke LB, Wara WM, Cohen BH and Boyett JM: Treatment of children with medulloblastomas with reduced-dose craniospinal radiation therapy and adjuvant chemotherapy: A Children's Cancer Group Study. J Clin Oncol *17*: 2127-2136, 1999.
25 Packer RJ, Sutton LN, Elterman R, Lange B, Goldwein J, Nicholson HS, Mulne L, Boyett J, D'Angio G, Wechsler-Jentzsch K, et al. : Outcome for children with medulloblastoma treated with radiation and cisplatin, CCNU, and vincristine chemotherapy. J Neurosurg *81*: 690-698, 1994.
26 Rutkauskiene G and Labanauskas L: Treatment of patients of high-risk group of medulloblastoma with the adjuvant lomustine, cisplatin, and vincristine chemotherapy. Medicina (Kaunas) *41*: 1026-1034, 2005.
27 Rood BR, Macdonald TJ and Packer RJ: Current treatment of medulloblastoma: Recent advances and future challenges. Semin Oncol *31*: 666-675, 2004.
28 WHO Pathology and genetics, Tumours of the nervous system. Lyon, France: IARCpress, 2000.
29 Ruas M and Peters G: The $p16^{INK4a}$/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta *1378*: F115-177, 1998.
30 Vogelstein B and Kinzler KW: Cancer genes and the pathways they control. Nat Med *10*: 789-799, 2004.
31 Kartner N, Riordan JR and Ling V: Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science *221*: 1285-1288, 1983.
32 Schinkel AH and Jonker JW: Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev *55*: 3-29, 2003.
33 Johnson DR, Finch RA, Lin ZP, Zeiss CJ and Sartorelli AC: The pharmacological phenotype of combined multidrug-resistance Mdr1a/1b- and Mrp1-deficient mice. Cancer Res *61*: 1469-1476, 2001.
34 Balayssac D, Cayre A, Authier N, Bourdu S, Penault-Llorca F, Gillet JP, Maublant J, Eschalier A and Coudore F: Patterns of P-glycoprotein activity in the nervous system during vincristine-induced neuropathy in rats. J Peripher Nerv Syst *10*: 301-310, 2005.
35 Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C and Gottesman MM: Targeting multidrug resistance in cancer. Nat Rev Drug Discov *5*: 219-234, 2006.
36 Chou PM, Reyes-Mugica M, Barquin N, Yasuda T, Tan X and Tomita T: Multidrug resistance gene expression in childhood medulloblastoma: correlation with clinical outcome and DNA ploidy in 29 patients. Pediatr Neurosurg *23*: 283-291; discussion 291-282, 1995.
37 Serrano M, Hannon GJ and Beach D: A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4 Nature *366*: 704-707, 1993.
38 Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS, 3rd, Johnson BE and Skolnick MH: A cell cycle regulator potentially involved in genesis of many tumor types [see comments]. Science *264*: 436-440, 1994.
39 Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K and Carson DA: Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature *368*: 753-756, 1994.
40 Ortega S, Malumbres M and Barbacid M: Cyclin D-dependent kinases, INK4 inhibitors and cancer. Biochim Biophys Acta *1602*: 73-87, 2002.
41 Al-Mohanna MA, Manogaran PS, Al-Mukhalafi Z, Al-Hussein AK and Aboussekhra A: The tumor suppressor p16(INK4a) gene is a regulator of apoptosis induced by ultraviolet light and cisplatin. Oncogene *23*: 201-212, 2004.
42 Ellison DW, Onilude OE, Lindsey JC, Lusher ME, Weston CL, Taylor RE, Pearson AD and Clifford SC: β-Catenin status predicts a favorable outcome in childhood medulloblastoma: the United Kingdom Children's Cancer Study Group Brain Tumour Committee. J Clin Oncol *23*: 7951-7957, 2005.
43 Neben K, Korshunov A, Benner A, Wrobel G, Hahn M, Kokocinski F, Golanov A, Joos S and Lichter P: Microarray-based screening for molecular markers in medulloblastoma revealed STK15 as independent predictor for survival. Cancer Res *64*: 3103-3111, 2004.
44 Kim JY, Sutton ME, Lu DJ, Cho TA, Goumnerova LC, Goritchenko L, Kaufman JR, Lam KK, Billet AL, Tarbell NJ, Wu J, Allen JC, Stiles CD, Segal RA and Pomeroy SL: Activation of neurotrophin-3 receptor TRKC induces apoptosis in medulloblastomas. Cancer Res *59*: 711-719, 1999.
45 Segal RA, Goumnerova LC, Kwon YK, Stiles CD and Pomeroy SL: Expression of the neurotrophin receptor TRKC is linked to a favorable outcome in medulloblastoma. Proc Natl Acad Sci USA *91*: 12867-12871, 1994.

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