Induction of drug resistance in embryonal rhabdomyosarcoma treated with conventional chemotherapy is associated with HLA class I increase*

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Effectiveness of conventional cytotoxic treatment of rhabdomyosarcoma (RMS) may be limited by the development of multidrug resistance (MDR) mediated by *mdr1* gene. This gene codes for P-glycoprotein (P-gp) which has been related to a immunoregulatory function. Modulation of HLA expression by P-gp has been described in different types of tumor cells including RMS. However, very little is known about biological implications of the P-gp expression in RMS patients treated with conventional chemotherapy. In order to study the problem, we used embryonal RMS tissue samples from treated patients. Our results indicated that positive RMS samples to *mdr1* show higher HLA class I expression than those which were negative to *mdr1* PCR, what indicates a significant correlation between the expression of both molecules. In addition, we developed two resistant RMS cell lines (A-204-1 and 2) using similar concentrations of actinomycin D as are plasma levels in clinical situation. Both resistant cell lines showed mdr1 expression and an increase of HLA class I expression which was dose-dependent. These results demonstrated that conventional chemotherapy of embryonal RMS is able to induce resistance which can modulate HLA class I expression and suggest that immunological studies of these tumors may be necessary to the design new specific therapeutic strategies.

Key words: HLA class I, P-glycoprotein, rhabdomyosarcoma, multidrug resistance, mdr1

Rhabdomyosarcoma (RMS) is the most frequent malignant tumor of mesodermal tissues in childhood and represents 4–8% of all pediatric malignancies. These tumors are roughly divided into three major subtypes: embryonal, alveolar and pleomorphic. The embryonal and alveolar subtypes represent the most common soft tissue sarcomas observed in children [1]. Throughout the years there has been a gradual improvement in survival of these patients, due to multidisciplinary treatment approaches including surgery, radiotherapy and especially chemotherapy. Drugs commonly used for the treatment include vinca-alkaloids, actinomycin D, alkylating agents and anthracyclines [2]. Although initial treatment is often successful, recurrences are not unusual, characterized by a poor response to cytotoxic treatment and by a significant increase of mortality [3]. There are some indications that the development of multidrug resistance (MDR) during and after the course of therapy is one of the major limitations in its treatment [4]. Resistance mediated by *mdr1* gene, which has been detected in RMS [5], is based on the expression of a 1280 amino acid transmembrane glycoprotein (170 kDa) termed P-glycoprotein (P-gp). Functionally, this molecule acts as an efflux pump of broad specificity, decreasing the intracellular drug accumulation that correlates with the extent of their resistance [6].

Multidrug resistance development mediated by P-gp is often associated with several changes in cell structure and metabolism of resistant cells, including the modulation of some antigen expression [7]. In RMS cells, the development of resistance increases the expression of classical markers of muscle differentiation such as myosin [8] and induces the apparition of myofilamentous material which is considered as a clear evidence of myogenic differentiation [9]. It has been demonstrated that this phenomenon also induces the modula-

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tion of HLA class I expression in RMS resistant cells in culture [10]. The clinical significance of the increase or decrease of HLA class I expression in tumor cells is not still clear although it has been related with prognosis and metastatic potential [11]. In fact, the MHC antigens downregulation has been recently associated with improved survival and antitumor immune response in lung and breast cancer, respectively [12, 13]. This finding indicates that the modulation of HLA antigen expression and its determination in the diagnostic and during the course of disease may be a prognostic factor in some tumors.

Resistance development and HLA expression are thus linked to the therapy response of patients with malignancies. For this reason, it seems appropriate to evaluate if the conventional chemotherapy used in embryonal RMS patients and the actinomycin D concentration in clinical use are able to induce resistance mediated by *mdr1* modulating HLA class I antigens expression in these tumor cells.

Patients and methods

Tissue samples and cell lines. RMS tumor samples were selected from the files of the Department of Pathology, Virgen de las Nieves Hospital (Granada), after the informed consent was obtained. For this study, we selected fourteen resected specimes which were classified as embryonal RMS in accordance with the histopathology described by NEWTON et al [14]. The mean age of the patients at the time of diagnosis was 6 years (range 2–22 years). There were nine male and five female patients. The anatomic site of the primary tumor included orbit (n=5), ear (n=3), maxilla (n=2), retroperitoneum (n=2), pelvis (n=1), buttock (n=1). All patients were treated with conventional chemotherapy (vinca-alkaloids, actinomycin D and alkylating agents). In addition, we used the embryonal RMS cell line A-204 which was obtained from the American Type Culture Collection (ATCC). This cell line was grown at 37 °C in an atmosphere containing 5% CO_2, with Dulbecco's modified Eagle medium (MEM) (Gibco, Grand Island, NY) supplemented with 10% FBS (Gibco), 20 mM L-glutamine, 3.5 mg/µl sodium bicarbonate, 4.5 g/l glucose, 250 U/ml ampicillin and 20 µl/ml streptomycin. Resistance in A-204 cell line was developed against actinomycin D according our laboratory protocol [9]. Cells were initially exposed to $0.6x10^{-9}$ mM actinomycin D. Dose levels in culture medium were then increased in steps to a similar concentration as are plasma levels in clinical situation [8], generating two resistant lines at $1x10^{-5}$ mM and $8x10^{-5}$ mM (A-204-1 and A-204-2, respectively).

RT-PCR analysis. Total RNA from residual paraffin embedded tissues were obtained according to CAO et al. [15]. Reverse transcription was done as follows: each tube contained a total volume of 100 µl, composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.01% gelatin, 800 µM each of the four deoxyribonucleotide triphosphates and 1 µM of *mdr 1* primers (sense primer: 5'ATATCAGCAGCCCACATCAT3' and antisense primer 5'GAAGCACTGGGATGTCCGGT3'). One microgram of total cellular RNA and two units of reverse transcriptase (Stratagene, La Jolla, CA) were added to individual tubes and the reaction was allowed to proceed at 42 °C for 45 min. Thermostable DNA polymerase (Pharmacia, Piscataway, NJ) was added to each tube (2.5 units), and *mdr1* product, if present, was amplified by PCR (94 °C for 1 min, 58 °C for 2 min and 72 °C for 3 min). PCR was carried out for 35 cycles with a final elongation step of 72 °C for 7 min. For analysis, 10 µl of the reaction product was run on a 2% agarose gel, visualized by ethidium bromide staining. The integrity of *RNA* was assessed by the amplification of *β-actin* mRNA (sense primer: 5'ATCATGTTTGAGACCTTCAA3' and antisense primer: 5'CATCTCTTGCTCGAAGTCCA5'). The images were scanned and analysed using a Bio-Rad documentation system (Quantity One Analysis Software). Relative *mdr1* mRNA expression was calculated as ratio of *mdr1/β-actin*.

Immunohistochemistry. Paraffin-embedded sections were deparaffinized and stained using the indirect peroxidase method as described FERNÁNDEZ et al [16]. The mAbs used were: W6/32 (1/50 dilution) against a common HLA class I heavy chain/ $ß_2$-microglobulin complex and GRH1 (1/50 dilution) against free $ß_2$-microglobulin (provided by Dr. F. Garrido, Virgen de las Nieves Hospital, Granada). The samples were incubated with the primary antibody-containing dilution for 30 min in a humid chamber at room temperature. For each sample, a peroxidase-conjugated secondary antibody identified the binding of the primary antibody. Antibody binding was visualized by incubation with PBS solution containing diaminobenzidine-tetrahydrochloride (Sigma, St. Louis, MO). The sections were counter-stained with Meyer's hematoxylin. The staining intensity was estimated independe ntly by two observers. Staining intensity was scored as 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. Appropriate positive and negative controls experiments were performed throughout.

Northern blot analysis. Total RNA was isolated from RMS cells by RNeasy kit (Qiagen, Alberslund, Denmark). Total RNA (10 µg) was electrophoresed on a 1.2% denaturing agarose gel and transferred overnight to a nylon membrane. Northern blot hibridization were performed using a ^{32}P-labell oligoprobe recognizing mdr1 sequences

(5'ATGGCGATGAAGACCAAGACGTATCAGGTG3'). The oligoprobe was radiolabeled with P^{32}-dCTP by random primer labeling using the Rediprime random labeling kit (Amersham Biosciences, Piscataway, NJ). After a 2 hr prehybridization step at 65 °C in a solution of 6x SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0), 5x Denhardt's solution, 100 µg/ml sheared salmon sperm DNA, and 0.5% sodium dodecyl sulfate (SDS), the blot was hybridized overnight in the same solution with 0.01M EDTA and the radiolabeled probe at a concentration of 20 ng/ml. After hybridization, the blot was washed successively in 2x SSC with 0.5% SDS, 2x SSC with 0.1% SDS and 0.1x SSC with 0.1%

SDS at 65 °C, and subsequently autoradiographed at –70 °C for 1–4 days. To control for loading differences, the blot was stripped with boiling 0.1% TE (10 mM Tris, 1 mM EDTA, pH 8.0) and rehybridized with a *β-actin* oligoprobe (5'-TGTTGGCGTACAGGTCTTTGCGGATGTCCA-3').

Cytotoxicity experiments To evaluate P-gp block, RMS cells ($2x10^5$/ml) were treated with different concentrations of actinomycin D in four replicate samples in the absence or presence of the verapamil at 10 μM for 1 h. After 72 h, cells were harvested by trypsinization and counted in a model ZBI Coulter Counter (Hialeah, FL). Cell viability was determined by trypan blue dye exclusion. The dose that inhibited 50% of growth (ID_{50}) was calculated from the curve for the percentage of cell survival at different concentrations of the drug.

FACScan analysis. Briefly, 10^6 RMS cells before and after treatment with verapamil were transferred to universal screw cap tubes containing phosphate-buffered saline (PBS), then washed and centrifuged at 225 g for 5 min. To determine HLA class I expression, the cells were fixed with 2% formaldehyde for 10 min at –20 °C and immediately washed three times in PBS at 4 °C. The cells were then incubated for 30 min at 4 °C with the two monoclonal antibodies (mAbs) W6/32 and GRH1. The rest of the procedure was done as described above. The results obtained were evaluated as mean fluorescence. The percentage increase in mean fluorescence was calculated by the formula: (MFI-MFB / MFB) x 100, where MFI is the mean induced fluorescence and MFB is the mean basal fluorescence.

Statistical analysis. Laboratory data were expressed as the mean ±SEM. Student's t-test was used to determine the level of significance. Sperman Rho correlation was used to examine correlations between mdr1 and HLA class I expression. Statistical analysis was performed using SPSS version 12.0 software.

Results

mdr1 mRNA expression in RMS tissue samples. mdr1 mRNA expression was determined by PCR. To demonstrate the integrity of the RNA preparations, PCR was performed using *β-actin* primers (Fig. 1A). Out of the fourteen RMS analysed, seven showed a clear positive PCR for *mdr1* with an *mdr1/β-actin* ratio between 2.08±0.4 and 0.34±0.09 (Fig. 1B), six of them were PCR negative and one of the RMS sample tissues was considered uncertain for *mdr1* PCR (Fig. 1A; line 11). In this sample, amplification of *β-actin* was very weak which could be caused by mRNA degradation.

HLA class I expression in RMS tissue samples. Inmunohistochemical analysis using the mAb W6/32 (Fig. 2) showed that out of the seven RMS samples positive for mdr1 PCR, five (71%) were clearly HLA class I positive and two of them negative. Four of the positive samples showed strong staining intensity (score 3) and the other one showed a moderate staining intensity (score 2) related to HLA class I expression. In contrast, HLA class I was predominantly negative in mdr1 negative tumours (six samples). A weak staining intensity (score 1) of HLA class I antigen was detected only in one out of the six RMS, and was absent (score 0) in the five (83%) mdr1 negative remaining cases. Similar results were found using the mAb GRH1 (data not shown). The uncertain sample for the mdr1 expression was clearly positive for HLA class I expression (strong staining). A positive correlation was found between HLA class I expression and *mdr1/β-actin* ratio (correlation coefficient = 0.973).

Mdr1/P-glycoprotein expression in A-204 cells. Transcripts of *mdr1* were detectable in both cell lines induced with actinomycin D and also in the parental cell line (Fig. 3A). However, studies of the bands, normalized by comparison with the *β-actin* signal of each sample, showed an elevated *mdr1* expression (six more times) in A-204-1 (7±1.5) and A-204-2 (6.8±1.3) in comparison to A-204 parental cells (1.2±0.3) (Fig. 3B). These results showed that the expression of *mdr1* was increased by exposure to the cells to actinomycin D.

Pharmacologic blockade of P-glycoprotein in A-204 cells. As shown in Table 1 the ID_{50} of both A-204 induced resistant cell lines (A-204-1 and A-204-2) was more than 30 times higher that in the parental line. In order to determine the degree of P-gp block with verapamil, we evaluated the modification of actinomycin D cytotoxicity in RMS cell lines. An approximately 12 and 20-fold increase in the effect of actinomycin D ocurred in A-204-1 and A-204-2 cells, respectively, after treatment with verapamil as compared to the ID_{50} values. Verapamil induced slight increase in drug cytotoxicity in A-204 parental cell line.

Table 1. Comparison of the concentration of drug needed to induce a 50% decrease in cell growth (ID_{50}) of RMS cells and effect to verapamil treatment on drug cytotoxicity

	a	b
A-204	$9x10^{-5}$ mM±0.5	$6x10^{-5}$ mM±0.4 (1.5)
A-204-1	$310x10^{-5}$ mM±4	$26x10^{-5}$ mM±0.5 (11.9)*
A-204-2	$870x10^{-5}$ mM±8	$43x10^{-5}$ mM±2 (20.2)*

The increase of drug cytotoxicity, indicated in parentheses, was calculated as the ratio between ID_{50} of the cell line treated with actinomycin D in the absence (a) and presence (b) of verapamil. All data are means ±SEM of four independent experiments. Significance of the differences (*$p<0.05$) was determined by statistical comparison (Student's t-test) of the values in the absence and presence of verapamil.

HLA class I expression and effect of the pharmacologic blockade of P-glycoprotein in A-204 cell lines. Analyses with the W6/32 mAb showed a significant increase in HLA class I expression in A-204-1 and A-204-2 resistant cell lines (111% and 137% increase in mean fluorescence respectively) when

compared with the parental A-204 cells (Fig. 4). Analyses with the GRH1 mAb confirmed the increase in HLA class I expression in both resistant cell lines (113% and 80% increase in mean fluorescence respectively) (Fig. 4). After treatment with verapamil, fluorocytometric analyses with the the W6/32 mAb showed a decrease of 46.5 and 57.3% in mean fluorescence intensity in A-204-1 and A-204-2, respectively. The decrease in the expression of epitope recognized by W6/32 was lower in A-204 cell line (16.2%) (Fig. 4). Analyses using GRH1 mAb confirmed the reduction observed in the expression of HLA class I with W6/32 mAb (52.6%, 62.9% and 18.8% decrease in mean fluorescence in A-204-1, A-204-2 and A-204, respectively).

Discussion

Although resistance to chemotherapy in embryonal RMS may be conveyed by different proteins, the overexpression of

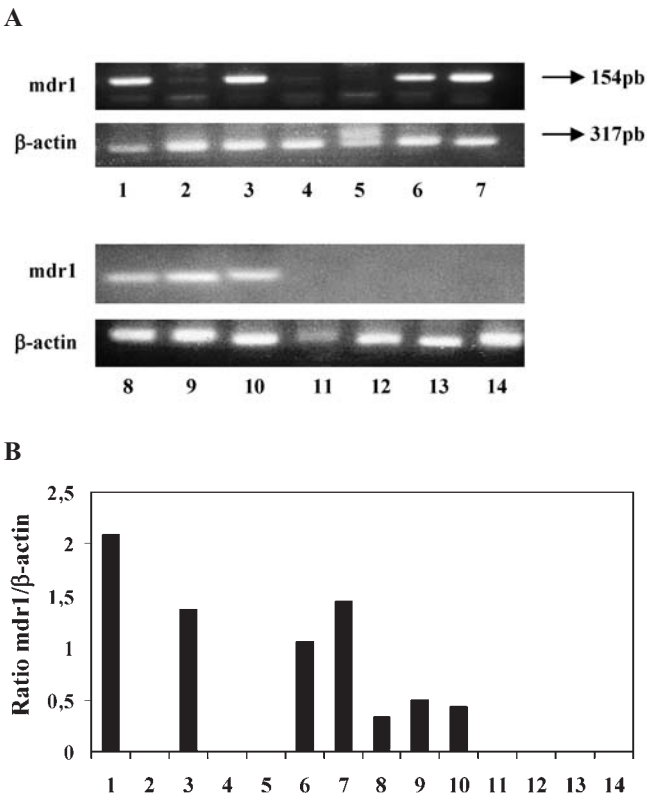

Figure 1. Reverse transcription polymerase chain reaction analysis (RT-PCR) of *mdr1* expression in embryonal RMS sample tissue. (A) Agarose gel electrophoresis with ethidium bromide staining of PCR products using primers for the amplification of *mdr1*. Using cDNA, obtained from mRNA that had been extracted from tissue samples, a band corresponding to *mdr1* was present in 7 of 14 RMS (lanes 1–14). Integrity of the RNA preparations was checked with a parallel PCR amplification of the cDNA using two specific primers for *β-actin*. (B) The band intensities of the PCR products of mdr1 were quantified and normalized to that of *β-actin* by densitometry.

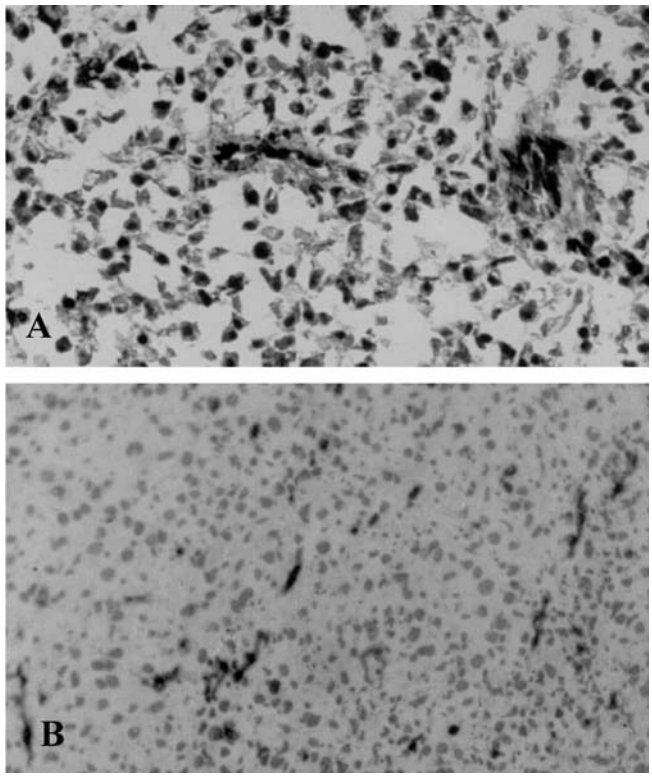

Figure 2. Example of immunohistochemical results of HLA class I expression in RMS tissues with mAb W6/32. (A) Marked HLA class I antigen expression revealed of a RMS tissue sample. (B) RMS showing a weak immunoreactivity. Magnification, x100.

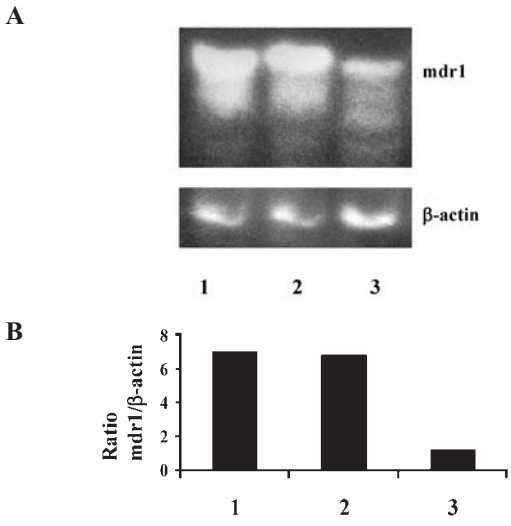

Figure 3. Northern blot analysis of *mdr1* expression in A-204 RMS cells. (A) A-204 resistant cells induced with actinomycin D (A-204-1: lane 1 and A-204-2: lane 2) showed an increase in the *mdr1* expression in comparison with the *mdr1* levels in A-204 parental cells (lane 3). Hybridization of the blot with *β-actin* was realized to demonstrate the integrity of the RNA preparations. (B) The band intensities of the PCR products of *mdr1* were quantified and normalized to that of *β-actin* by densitometry.

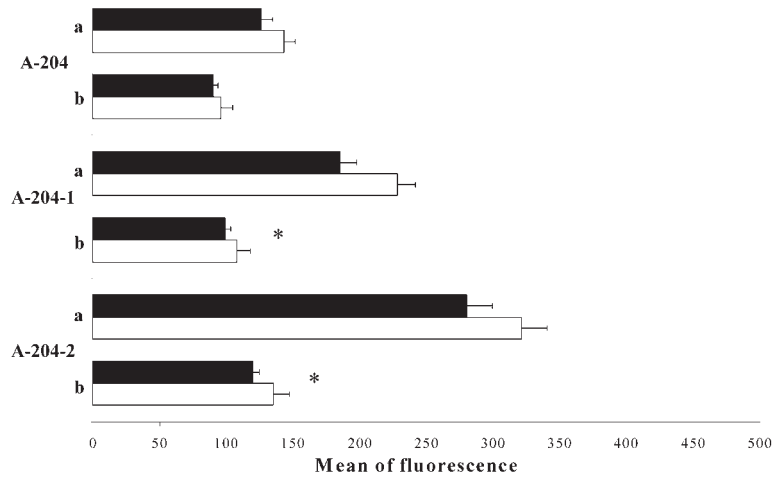

Figure 4. Analysis by FACScan of HLA class I antigen expression in A-204 RMS cell lines before (a) and after (b) exposure of cells to verapamil at a nontoxic concentrations (10 µM) for 1 h. The expression of HLA Class I was determined using the mAbs W6/32 (■) and GRH1 (□). Results are expressed as mean fluorescence and are representative of four independent experiments. (*p<0.01).

the *mdr1*/P-gp is one of the most frequent mechanism detected in this type of tumors [17]. However, the biological relevance of the P-gp modulation in embryonal RMS are discussed [18, 19, 20].

It has been demonstrated that one of the implications of P-gp expression in tumor cells are the modulation of some antigens due to its homology with putative peptide transporters TPA [21]. A correlation between P-gp and MHC expression has been clearly demonstrated in hematologic malignancies. Resistance mediated by *mdr1* modulated HLA expression in acute myeloid leukemia and in cell lines derived from human leukemia/lymphoma [22, 23]. MDR phenotype in leukemia cells (L1210) was related to a fourfold up-regulation of MHC class I molecules [24]. Experimental finding in breast and colon cancer showed that the transfection of the mdr 1 gene and its posterior induction to the expression was associated with a higher expression of MHC class I molecules [25]. In RMS, induction of MDR had an effect on the differentiation, inducing a modulation in their antigens expression [8]. This change may be related to the therapeutic response of the tumor, because it has been observed that patients with recurrences of well differentiated RMS have a poor prognosis [20, 26]. Recently, we have demonstrated that low doses of actinomycin D ($1.2x10^{-6}$ mM) were able to induce resistance in RMS cells in culture in which modulation of MHC expression took place [10]. This modulation could be physiologically and clinically relevant since a quantitative alteration in MHC antigens have been associated with modifications in the aggressive biologic potential of some tumors [27]. However, we do not know if this modulation of MHC antigens also takes place in RMS patients treated with conventional chemotherapy. In order to analyze this hypothesis, we have selected a group of embryonic RMS samples from patients treated with classical cytotoxic therapy. A 54% of the analyzed RMS showed mdr1 expression. Similar findings were found by KOMDEUR et al [5] in a group of fourteen RMS, although in this study P-gp was determined in embryonal, alveolar and pleomorphic RMS by inmunohistochemistry. Our results indicated that positive RMS to *mdr1* showed higher HLA class I expression than those which were negative to *mdr1* PCR, which seems to prove a significant correlation between the expression of both molecules. This finding shows that the conventional treatment of RMS may induce modulation of MHC antigen expression associated to the development of resistance. Moreover, actinomycin D therapeutic doses (10 mM and $8x10^{-5}$ mM) were used to induce resistance in A-204 RMS cells. The resistant A-204-1 and A-204-2 cell lines showed an increase in HLA class I expression higher than other RMS resistant cell lines (RD-DAC and T-32-DAC) induced with lower actinomycin D doses [10]. A non-toxic treatment with verapamil confirmed the relationship between *mdr1*/P-gp and HLA in these cells. These results suggest that MHC modulation in RMS is dose-dependent and that may be related to the degree of mdr1 expression.

The importance of HLA class I expression on established tumors has been demonstrated since a prerequisite for tumor eradication by cytolytic T cells is the recognition of tumor antigen presented by these molecules [27]. Resistant tumor cell lines derived from human leukemia/lymphoma with a different expression of MHC molecules, showed modified susceptibility for immunotherapy [20]. This finding suggests that the modulation of the MHC antigens induced by the development of resistance may be related to the antitumour immune response. In fact, it has been recently shown that the down-regulation of HLA class I antigens found in non-small cell lung cancer was associated with improved survival [12]. Although further studies will be necessary, the modulation of MHC in RMS treated with conventional therapy suggests that immunological studies of these tumors may assist in the design of specific therapeutic strategies that complement current chemotherapy treatments.

In conclusion, we have demonstrated that conventional therapy of RMS patients was able to induce resistance mediated by *mdr1* and that the development of this resistance mechanism was related to the increase in the HLA class I expression, which may be relevant to the application of new immunotherapy strategies.

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