Orbital Rhabdomyosarcoma: Relationship Between DNA Ploidy, P53, Bcl-2, MDR-1 and Ki67 (MIB1) Expression and Clinical Behavior

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Abstract. *Background: As for rhabdomyosarcoma (RMS) of other anatomic regions, the evaluation of traditional clinicopathological parameters does not allow the unequivocal outcome prediction of the single cases of orbital RMS. We investigated the role of DNA ploidy and immunohistochemical expression of p53, bcl-2, MDR-1 and Ki67 (MIB1) in the prognostic evaluation of orbital rhabdomyosarcomas. Materials and Methods: The study population consisted of 11 selected cases. Serial sections of each tumor, stained with Feulgen's technique, were analyzed for the DNA content, using the QUANTIMET 500c Leica analyzer, QWINVO200A software. The results were compared with the immunohistochemical expression of p53 (wild plus mutated, W&M and mutated), bcl2, MDR-1 and Ki67 (MIB1), and with follow-up data. Results: The statistical analysis of results showed that the cases of tetraploid and/or multiploid RMS, overexpressing p53 (W&M and mutated) and MDR-1, were characterized by an overall worse prognosis. On the contrary, the tumors with a favourable clinical course showed hyperexpression of MIB1 and absence of mutated p53 expression. Significantly higher MIB1 expression was found in the relapse-free group of tumors, with respect to the RMS with relapse (both in primary tumors and relative relapses, $p<0.05$). This finding could justify the higher sensibility to pharmacological therapy of RMS of the first group. The group of RMS with a worse prognosis (primary tumors and relapses) showed instead p53 overexpression (W&M and mutated), MDR-1 expression and multiploidy, with high 5cEE values and tetraploid peaks. No significant difference was found concerning the expression of bcl-2 among the two groups of RMS ($p>0.05$). Conclusion: The evaluation of DNA ploidy, p53, MIBI and MDR-1 expression could be used for subtyping of orbital RMS into two prognostically different subcategories, respectively RMS responder to the therapy, with favourable clinical outcome, and RMS with a worse prognosis, requiring more aggressive therapeutic protocols.*

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Key Words: Orbital rhabdomyosarcoma, p53, bcl2, MDR-1, Ki67 (MIB1), DNA ploidy, prognosis.

Rhabdomyosarcoma (RMS) represents 19% of soft tissue sarcomas (1) and almost 45% of juvenile sarcomas. It originates from primary mesenchymal tissue and shows skeletal muscle differentiation (1-3). RMS localized in the orbital region is characterized by a relatively better outcome with respect to RMS of other body sites (1, 2). However, the evaluation of traditional clinicopathological parameters does not allow the unequivocal prognostic prediction. Many attempts have been made recently in order to find new reliable factors predictive of outcome (2-13).

We evaluated the immunohistochemical expression of p53 protein (wild plus mutated, *W&M* and mutated), bcl-2, MDR-1 and MIB1 (Ki67) on a selected series of orbital RMS. The findings were compared with those concerning DNA ploidy status and with the follow-up data of the patients. The aim of this study was to investigate their role as prognostic markers in orbital RMS.

0250-7005/2004 $2.00+.40

Table I. *List of primary antibodies.*

Specificity	Clone	Dilution	Producer
MIB1	Ki-67 antigen, IgG1	1:150	Immunotech
Wild & mutant p53	P53, DO-7, IgG2b, kappa	1:100	DAKO
Mutant p53	P53, mutant, aa256-270	1:100	Serotec
Bcl-2	124, IgG1 kappa	1:80	DAKO
MDR-1	P-glycoprotein, UIC2, IgG1	1:4	Immunotech

Materials and Methods

Selection of cases. From the files of the Department of Biomorphological and Functional Sciences, Pathology Section of the "Federico II" University, Faculty of Medicine and Surgery, Naples, Italy, 11 cases of surgically excised RMS of the orbital region, between January 1980 and December 1997, were retrieved. All the cases were clinically treated and followed respectively at the Orbital Unit of the Department of Ophthalmology and at the Department of Pediatrics of the "Federico II" University of Naples, Italy. Only cases with complete clinical follow-up data and a tumor-size less than 5cm were included in the study. Anatomical site, presence of relapses and/or metastasis and the eventual neoadjuvant or adjuvant therapy performed were recorded for each case. According to the follow-up data, patients were subdivided into two sub-groups, respectively without and with relapses. Sections stained with H&E were used to confirm the original diagnosis.

Immunohistochemistry. Four-μm-thick serial sections of each case, from formalin-fixed, paraffin-embedded tissue, were mounted on polylysine pre-treated slides. In order to help the binding to the antigens, pre-incubation in a microwave oven for 15 minutes (3 cycles of 5 minutes), in citrate buffer 10 mM at pH6, was carried out. Endogenous peroxidases were quenched by incubation for 20 minutes in 0.3% hydrogen peroxide in methanol. In order to avoid non-specific binding of antibodies, sections were then pre-incubated with non-immune mouse serum (1:20; Dakopatts, Hamburg, Germany) diluted in PBS/BSA (1%) for 25 minutes at room temperature. After washing twice with Tris-HCl buffer, primary antibodies were applied overnight in a damp-room, at 4°C. The primary antibodies used were: anti-Ki67 (MIB1), anti-p53 protein (respectively W&M and mutant oncoprotein), anti-bcl-2 oncoprotein and anti-MDR-1 (multidrug resistance protein) (Table I). A positive control specimen accompanied each staining-run to monitor the accurate performance of the reagents (human palatine tonsils for MIB1, high-grade infiltrating breast cancer for wild & mutant and mutant p53 oncoprotein, a reactive lymph node for bcl2 oncoprotein and a normal intestinal mucosa for MDR-1 protein). Negative controls were also performed for all the antibodies in each run, substituting the primary antibodies with non-immune serum (DAKO Antibody Diluent, Dakopatts, Hamburg, Germany). Then, the streptavidin-biotin system (DAKO LSAB, HRP) was applied. Staining was completed with incubation with 3,3'-diaminobenzidine (DAB, Vector Laboratories, Burlinghame, USA), followed by slight nuclear counterstaining with Mayer's haematoxylin. Only for bcl-2, a double passage with secondary and tertiary antibody was performed (14). The results were evaluated separately by two observers (R.F., S.S.) unaware of the clinicopathological and follow-up data of each case.

For each case, at least 400 neoplastic cells were evaluated as follows: Ki67 (MIB1) positivity was expressed as a percentage; for the other antibodies, a semiquantitative evaluation of the immunohistochemical staining was performed, according to an arbitrary range from 0 (absence of immunopositivity), to + (slight,<30%), ++(moderate,<50%) and +++ (strong positivity,>50%). For mutant p53 only, the reaction was simply judged as "negative" (≤5% of positive cells) or "positive" (>5% of positive neoplastic cells).

DNA ploidy. A 4-μm-thick section of each case was dewaxed through serial passages from xylene to decreasing alcohols, then stained with Feulgen's (fuchsine sulphuric acid) technique, that specifically binds nuclear DNA. DNA quantitative analysis was made by computerized analysis, using hardware QUANTIMET 500 C analyzer, LEICA, connected with a CCD camera (SONY) and a LEITZ ORTHOPLAN microscope. The software used was the QWINVO200A (LEICA).

For each case, DNA content was measured in 400 neoplastic cells, in random selected fields from the most representative areas of the lesion. The DNA content of at least 30 lymphocytes was measured in each section to establish the individual 2c (diploid) reference value. The following indices were determined:
1) normal diploid DNA content (2c);
2) 2c deviation index (2c DI), defined as the average deviation with respect to the diploid value;
3) events exceeding 5c, defined as the percentage of neoplastic cells with a DNA content higher than 5c;
4) DNA malignancy grade (DNA-MG, a logarithmic transformation of the 2c value to produce a continuous scale ranging from 0 to 3.0 DNA content).

Following widely accepted criteria, histograms were classified according to the prevalent tumor cell population as follows:
- diploid: tumors in which more than 70% of cells show DI from 0.8 to 1.12.
- tetraploid: >20% of tumor cells with DI from 1.8 to 2.12 and >5% with D l from 2.12 to 4.12.
- aneuploid: >10% of tumor cells with DI distributed in all other values and/or >20% of tumor cells with DI from 1.12 to 1.8 (15).

Statistical analysis. Data were analyzed using SPSS software, 6.1 version for Windows. All the relevant clinicopathological and immunohistochemical data and DNA ploidy individual parameters were considered. Univariate analysis was performed using the Mann-Whitney *U*-test for comparing primitive tumors and the corresponding relapses. The Cox proportional hazard regression - model was used to compare the data concerning relapsed tumors with the relapse-free ones and, for multivariate analysis, in relation to the presence/absence of relapse and to the survival rate at 2 and 5 years. Only variables that were significant at univariate analysis ($p<0.05$) were used for multivariate analysis. Only values of $p<0.05$ were considered as significant. The significance of correlations was evaluated by applying the Spearman correlation test.

Results

Subjects. The study population consisted of 11 subjects (5 boys and 6 girls), consecutively recruited from January 1984 to July 1999, from a population of 19 subjects referred to the Department of Ophthalmology of the Federico II

Table II. *Clinical and pathological variables of 11 cases of orbital rhabdomyosarcomas.*

Relapsed cases of RMS

Patient	Sex	Age (yrs)	Histotype	Follow-up (months)	Therapy
1	M	4	Embryonal	192 (1R) □	Yes
2	F	2	Alveolar	5 (2R) □	Yes
3	F	4	Embryonal	1 (1R) □	Yes
4	M	12	Embryonal	6 (3R) ◊	Yes
5	F	6	Embryonal	2 (1R) □	Yes
6	F	14	Embryonal	24 (1R) □	Yes

Not relapsed cases

Patient	Sex	Age (yrs)	Histotype	Follow-up (months)	Therapy
1	M	5	Embryonal	192 □	Yes
2	M	7	Embryonal	144 □	Yes
3	F	16	Embrional	48 □	Yes
4	M	1	Embrional	84 □	Yes
5	F	16	Alveolar	72 □	Yes

M=male; F=female
N°R=number of relapses
◊=dead; □=alive

Table III. *Immunohistochemical findings in 11 cases of orbital rhabdomyosarcomas.*

Relapsed cases of RMS

Patient	MIB-1	Bcl-2	W & M p53*	mp53°	MDR-1	DNA ploidy
1	30%	+	+++	-	++	A+t
2	40%	-	+++	+	++	A+t
3	20%	+++	++	+	++	A+t
4	20%	-	+	+	+	A
5	35%	-	+	+	++	A+t
6	30%	-	++	-	++	A

*W& M = wild plus mutated form
°mp = mutated p53

Cases without relapses

Patient	MIB-1	Bcl-2	p53 w-m	mp53	MDR	DNA ploidy
1	50%	+	-	-	-	A
2	50%	+++	-	+	-	A
3	60%	-	-	-	-	A
4	40%	-	++	-	+	A+t
5	60%	+	-	-	-	A

A: aneuploidy; t: tetraploid

University of Naples, Italy, for orbital RMS. Only the cases for which a complete clinical follow-up was available (of between 2 and 15 years) were considered for the study. The mean age of the selected patients was, at the time of diagnosis, 8 years (range: 1-16 years).

Six patients developed one or more local relapses, the first one occurring 1 to 1.6 years from diagnosis. A second group (5 patients) did not show post-surgical relapses during the observation time. In the first group (2 boys and 4 girls, age range 2-14 years), the tumor location was in 4 cases in the right orbital region and in 2 cases in the left orbital region. In the second group (3 boys and 2 girls, age range 1-16 years), the tumor site was in 3 cases in the right orbital region and in 2 cases in the left orbit.

Two patients (one of the first and one of the second group) were treated with adjuvant radiotherapy (total of 6000 cGy) after diagnosis. The remaining patients were all treated with radio- and poly-chemotherapy after diagnosis. The total amount of radiation used ranged from 4500 to 6000 cGy, (200 cGy at a time), five times per week. Chemotherapy treatment consisted of vincristine (2mg/m^2) and vincristine + actinomycin D (0.4 mg/m^2), on alternate weeks. A further chemotherapeutic protocol consisted of a monthly administration of vincristine, adriamycin, cyclophosphamide alternating with vincristine, cyclophosphamide and dactinomycin, for one year in case of relapse. At pathological evaluation, five cases of the first group and four cases of the second group showed an embryonal histotype, sometimes with partial condroid differentiation (two cases of the first and one case of the second group); the remaining one case of the first and one of the second group showed an alveolar histotype. One of the patients of the first group relapsed and died from disease. In the remaining cases, the first relapse showed an embryonal histotype, with partial condroid differentiation in two cases. The further relapses occurred in two patients, showed the following histotypes: embryonal, embryonal with partial condroid differentiation and alveolar with solid aspects. The treatment of the relapses consisted of local radiotherapy (doses of 6000 cGy) *plus* the same poly-chemotherapeutic protocol performed after the first diagnosis; two patients underwent exenteratio orbitae.

The clinicopathological variables of the population studied are reported in Table II.

Immunohistochemistry. The immunohistochemical findings of RMS of this study are reported in Table III.

Ki67 (MIB1). The difference among the extent of nuclear MIB1 expression was statistically significant in discriminating between the two groups of RMS of the study, with nuclear immunopositivity for MIB1 higher in relapse-free cases (20-

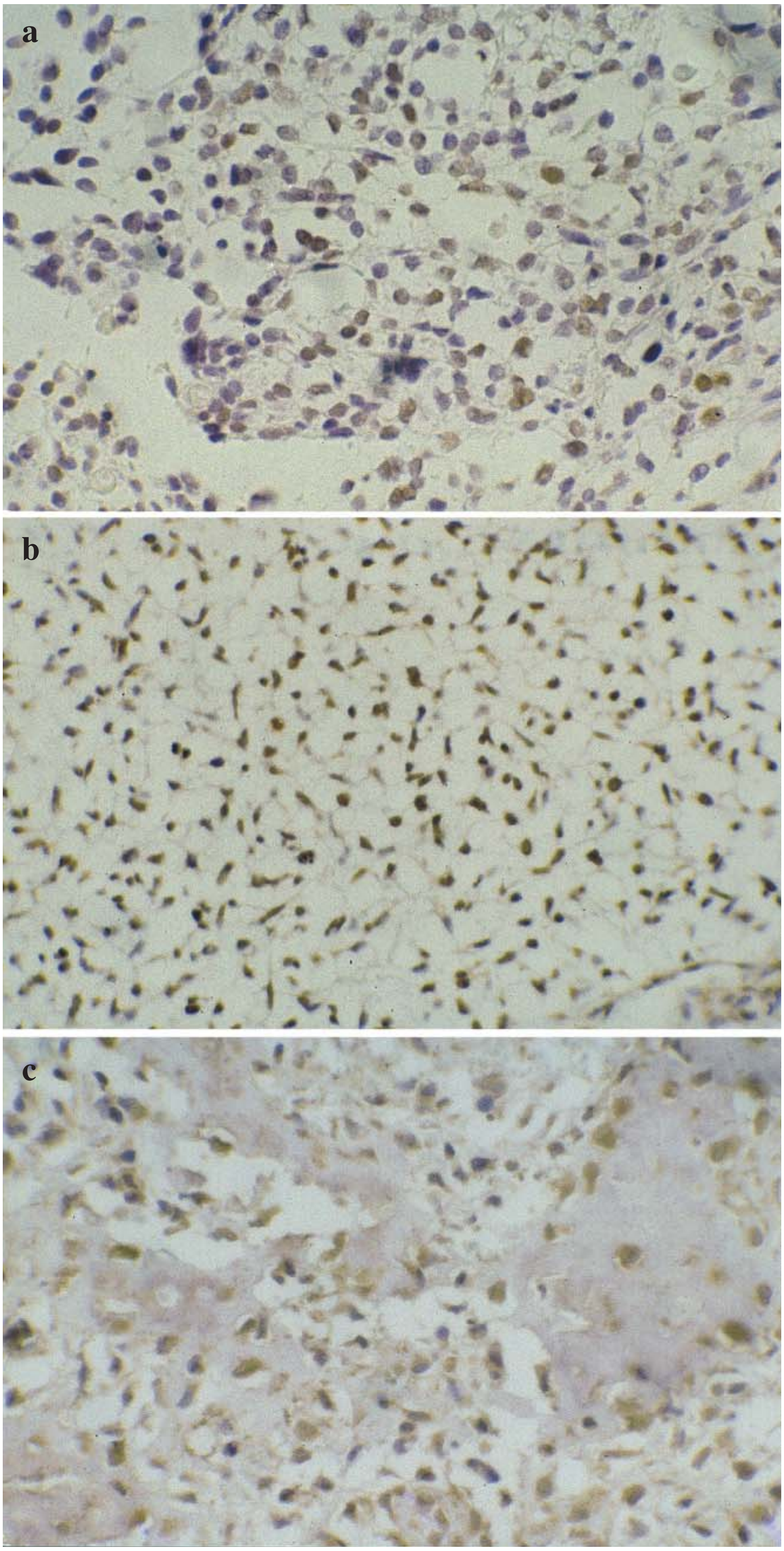

Figure 1. *a. Moderate Ki67 (MIB1) expression in one relapsed case (LSAB, 150x). b. Diffuse, strong nuclear Ki67 (MIB1) expression in one case of relapse-free orbital RMS (LSAB, 100x). c. Presence of bcl-2 protein in one relapsed case of orbital RMS with chondroid differentiation (LSAB, 250x).*

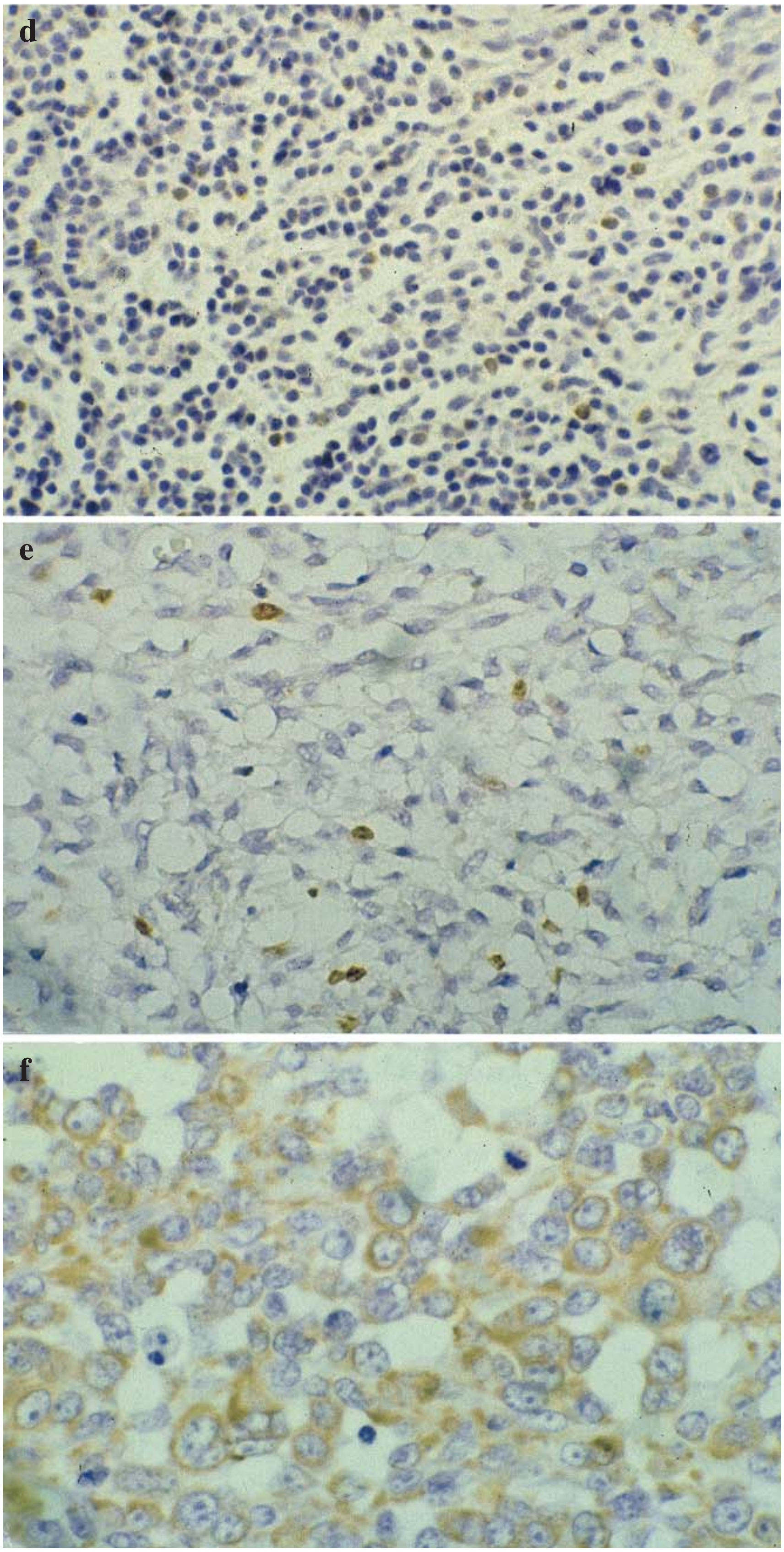

Figure 1. *d. Low expression of bcl-2 in one relapse-free case of orbital RMS (LSAB, 400x). e. Expression of mutated p53 in one relapsed case of orbital RMS (LSAB, 250x). f. Definite, strong expression of MDR-1 in one relapse-free case of orbital RMS (LSAB, 400x).*

40% of positivity in the first group; 40-60% in the second group, $p<0.05$ (Figures 1a, b). No significant difference in MIB1 expression was found between the relapses and relative primitive tumors ($p>0.05$).

Bcl-2. No detectable immunohistochemical cytoplasmatic expression of bcl-2 was found in four cases of the 1st group (recurring RMS) and in two cases of the 2nd group (relapse-free RMS); a slight expression was detected in one case of the 1st group (Figure 1c) and in two cases of the 2nd group, and a strong expression in one case of the 1st group and in one of the 2nd group (Figure 1d). No significant difference ($p>0.05$) was found comparing bcl-2 expression in the two groups of tumors, or between relapses and primitive RMS.

P53 protein. P53 wild plus mutated (W&M). The nuclear expression of the W&M p53 protein was found significantly higher in the 1st group with respect to the 2nd one, being low in two cases, moderate in two and strong in two cases of the 1st group, but absent in four cases and moderate in the remaining one case of the 2nd group. A statistically significant association was detected between wild and mutated p53 overexpression and the more aggressive orbital RMS forms ($p<0.05$).

Mutated p53. The mutated form of p53 protein was expressed in 4 cases of the 1st group (Figure. 1e) and only in one case of the 2nd group. This was statistically significant in discriminating between the two groups of tumors ($p<0.05$).

MDR-1. MDR-1 protein showed: low cytoplasmic positivity in 1 case of the relapsing group of RMS and in one of the non-relapsing group; moderate/strong positivity in the remaining five cases of RMS with relapses (Figure 1f); absent MDR-1 expression was found in the remaining four cases of non-relapsing RMS. The statistical analysis showed that the MDR-1 expression was able to discriminate between the two groups of RMS ($p<0.05$). All the relapses showed positivity for MDR-1 protein, ranging from low to moderate to strong; this did not reach statistical significance in discriminating between primary tumors and relapses ($p>0.05$).

DNA ploidy. Aneuploidy was found in all cases of both groups of RMS examined. The statistical analysis of differences among the DNA ploidy parameters considered for the study showed that DNA Dl, 2cDI and DNA MG were unable to discriminate between the two groups of RMS, or between primary tumors and relapses. However, considering the DNA ploidy histogram profiles, a statistically significant incidence of tetraploid peaks was found in the 1st group with respect to the 2nd one ($p<0.05$). This finding was also observed in all the relapses of patients of the first group, with the only exception being the unique case of the third relapse, which showed hypertetraploidy. A trend to statistical significance between relapses and high values of 5cER was also observed.

Discussion

The last 25 years have seen dramatic advances in the treatment of childhood sarcomas, particularly of RMS. However, there is still a conspicuous number of patients that fail to respond adequately to therapy. One of the factors thought to be responsible for this therapeutic failure is the characteristic heterogeneity of childhood sarcomas, which make the identification of appropriate therapies for each subtype of tumors difficult (16).

Multidisciplinary therapeutic protocols have often proved useful in the treatment of these heterogeneous neoplasia. In particular, protocols including a more intense chemotherapic regimen and a more sophisticated radiotherapy, combined to eventual advanced surgery, allowed the elimination of high-dose radio-treatments and/or of demolishing surgery (16, 17). This led to an increase of the survival probabilities of patients from 1:5 in the 1960s to the current 3:4 ratio (16). However, the improvement of therapeutic response was not linear and many aspects of the individual difference in patient outcome still need to be cleared up. In particular, patients with RMS of the head and neck region, which constitutes 40% of paediatric RMS, show a huge variability in survival (18,19). In fact, even if a significant correlation with the specific onset district has been shown for tumors of this anatomic site, with orbital lesions generally showing the best clinical evolution, in the restricted field of orbital RMS the response to therapy of the single cases still remains unpredictable, based on the current classification schemes (18-20).

The first widespread RMS classification (Horn and Enterline) was based on clinicopathological features and identified four histological subtypes of RMS: embryonal, botrioid, alveolar and pleomorphic (10). Later, WHO added to this classification the spindle cells subtype (12). The Intergroup Rhabdomyosarcorna Study (IRS) and especially the International Society for Pediatric Oncology (SLOP) demonstrated a worse prognosis for alveolar rhabdomyosarcoma with respect to the other histotypes (7).

Furthermore, the National Cancer Institute (NCI) introduced a new RMS subcategory, the solid/alveolar histotype, whose prognosis was superimposable on the classical alveolar histotype (13). Among these various classifications, the proposal of two main groups of RMS, respectively with favorable and unfavorable prognosis, according to the presence/absence of alveolar growth pattern, offered, of course, a possibility of differentiating these neoplasms on a prognostic basis (8).

In recent years, the introduction of additional clinicopathological parameters correlated with the outcome of this neoplasia led to the formulation of the International Rhabdomyosarcoma Classification (IRC). Eight hundred cases of RMS were classified by expert pathologists, following the different schemes. The histological subtype was strictly correlated with the global survival time, so that the traditional Horn-Enterline scheme was modified and enriched with elements taken from the SLOP and NCI classifications (11). As previously outlined, besides morphological parameters, a series of clinical factors influencing the prognosis, such as age, site, local invasiveness and resection completeness, were taken into account (16).

However, at present no classification and treatment scheme are able to give definitive solutions with regard to the prognosis of single cases of RMS. This has led to the hypothesis that RMS should be classified by also taking into account their biological basis, in order to better predict their clinical behaviour (16). In particular, this seems the case for orbital RMS, considering the absence of peculiar clinicopathological features able to discriminate cases that developed relapses and/or death from those with a more favorable outcome.

The analysis of the results of our study led us to formulate some considerations. The first concerns the value of the proliferation index (P.I.) in discriminating between the two groups of orbital RMS. As show in the Results section, a statistically significant higher proliferation index evaluated by the immunohistochemical expression of Ki67 (MIB1) was found in the relapse-free group of tumors, with respect to that found in the primary tumors and relative relapses of the first group of RMS of our series. This finding may constitute the molecular basis of the higher sensitivity to therapy of the RMS characterized by high PI, according to what has been shown in other types of solid tumors (21).

The second consideration emerges from the analysis of the data concerning DNA ploidy, which showed in the relapsed group of RMS the presence of multiploidy with a trend to higher 5cEE values. This finding testifies the presence of high clonal heterogeneity in the group of RMS characterized by a worse prognosis, with tetraploid peaks persistent in relapses, which finally acquired a real tetraploid profile. These data are in agreement with previous reports signaling tetraploidy as an unfavorable prognostic factor in RMS (22-24). In human tumors aneuploidy is generally associated with a worse outcome; however, in some tumor types, as RMS, a better response to chemo- and radiotherapy in case of aneuploidy has been frequently recorded (25-28). In particular, the frequent association between unfavorable prognosis of RMS and diploid or tetraploid DNA content has been reported. On the contrary, the cases of RMS with favorable prognosis have often been associated to a hyperdiploid pattern (16, 29-31).

The observed reduction of the number of DNA peaks, with the prevalence of the tetraploid peak in relapses, suggests a possible therapy-related effect of clonal selection. For that concerning the expression of p53 oncoprotein in RMS (as well as in leiomyosarcomas), the frequent presence of p53 mutation at level of codon 245 has been registered, a hot-spot shared also by carcinomas of various anatomical districts (32). The data of this study showed a significant association between the presence of immunohistochemical positivity to p53 (W&M *plus* mutated p53 form) in RMS with recurrence(s) with respect to recurrence-free tumors. Moreover, the p53 overexpression (both W&M and mutant forms) in our series of cases significanly correlated with the more aggressive histotypes of orbital RMS (alveolar, solid-alveolar and embryonal with chondroid differentiation) ($p<0.05$) (33). This is in full agreement with the widely accepted role of tumor suppressor genes, of which p53 is the prototype, in regulating cell cycle and, when mutated, in the process of carcinogenesis (34-36). As is well known, p53 is the most frequently transformed suppressor gene in human tumors (36). In normal cells, it provokes the arrest of cell cycle in G1, in reply to genotoxic damage, favoring an efficient protection of DNA, before duplication. In the G1- phase, the p53-mediated block of cell cycle is principally carried out by the p2l protein, a CDKs inhibitor responsible for the entrance into the S-phase, which is activated by the protein product of p53 gene. Cells that lack p53 function are not capable of stopping in the G1-phase, following the damage caused by chemo/ radiotherapic agents (37,38).

A lack of statistical significance emerged to the contrary concerning the expression of bcl-2 protein in our series of orbital RMS.

The protooncogene bcl-2 codifies for an intracellular protein which protects cells from programmed death (apoptosis). The gene that codifies for bcl-2 is expressed in different cell types, mainly in cellular compartments characterized by a long life-span interval. High expression of bcl-2 correlates with an increase of cellular life, due to reduction of apoptosis, through an inhibition of functions triggering the activation of the cellular cycle. Abnormally high levels of bcl-2 have been found in a great number of human tumors (39,40). This overexpression generally correlates with some biological characteristics of the tumor, such as a low level of cellular proliferation and a high proportion of cells in G0-phase. No significant difference in bcl-2 expression was found in the two groups of RMS of our study, nor between the relapses and primitive tumors. These results are in agreement with similar findings concerning other childhood sarcomas (40).

Finally, the last consideration concerns the value of MDR-1 expression in our series of orbital RMS. As is well known, the MDR-1 (multidrug resistance, Pgp protein) enables the tumor cells to protect themselves from multiple, unrelated drugs. The MDR-1 and MDR-1-associated proteins (MRP), members of the ATP-binding cassette (ABC) transporters family, were identified in human tumors, in particular in leukemias, myelomas and childhood neoplasias. The presence of MDR-1-expressing clones could justify tumor resistance to mono-drug-based chemotherapy and the poor outcome of some treated tumors. However, inclusion of a reversing or chemosensitizing agents such as verapamil or cyclosporin A has improved response to chemotherapy. Doxorubicin is the major chemotherapeutic agent used in the treatment of RMS, as well as in the majority of sarcomas in children and adults. However, the expression of MDR-1 protein, a membrane pump, induces *in vitro* resistance to anthracycline-family drugs (41). Doxorubicin is also used in cases of embryonal or alveolar RMS with unfavorable clinical behaviour (16). Tumors expressing MDR-1 are variously doxorubicin-resistant, so the immediate identification of drug resistance in a primary tumor could help the therapeutic programming (41). Analysis of the data concerning the MDR-1 protein expression in our series of orbital RMS showed a definite association with the subgroup of RMS with a worse prognosis, with a tendency to be associated to p53 immunopositivity (both wild/mutant and mutant) ($p<0.05$). These findings were confirmed by multivariate analysis, which showed the association of a worse outcome with the subset of orbital RMS characterized by high expression of p53 protein (wild and mutated *and* mutated), aneuploidy with high values of 5cEE and expression of MDR-1 protein.

The results of this study, if confirmed by further data on a larger series of cases, indicate that the combined evaluation of p53 and MDR-1 protein expression and the DNA ploidy status constitute a promising tool for the biological subtyping and therapeutic management of orbital RMS.

Acknowledgements

This study was supported by a grant from the National Council of Research (CNR), Italy. The authors thank Mr. Armando Coppola, Mr. Ferdinando Stumpo and Mr. Pasquale Signoriello for their valuable technical assistance, and Mrs. Patricia Reynolds for the English editing of the manuscript.

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Received March 24, 2003
Revised September 26, 2003
Accepted October 20, 2003