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Characterization of a New Human Embryonal Rhabdomyosarcoma Cell Line, RMS-GR

Juan Emilio Fernández,[1] Jose Prados,[2] Consolación Melguizo,[2] Nicola Arena,[3] Fabio Malavasi,[4] Luis Álvarez[1] and Antonia Aránega[1, 5]

[1]Basic Cardiovascular Research Section, Department of Morphological Sciences, School of Medicine, University of Granada, 18071 Granada, Spain, [2]Department of Health Sciences, University of Almería, 04002 Almería, Spain, [3]Histology Institute, School of Medicine, University of Degli Studi di Sassari, 07100 Sassari, Italy and [4]Laboratory of Cell Biology, Department of Genetics, Biology and Medical Chemistry, University of Torino, 10126 Torino, Italy

A human tumor cell line designated RMS-GR was established from an embryonal rhabdomyosarcoma. The monolayer cells were polygonal, round or spindle-shaped. The RMS-GR cell line became stable with a doubling time of 42 h. Tumorigenicity of the cells was confirmed by heterotransplantion into nude mice. Electron microscopic images showed typical cytoplasmic inclusion of aggregated intermediate filaments and myofibril-like thin filaments. The expression of desmin, vimentin, actin and human myoglobin was recognized by cytofluorometric analyses, and a large fraction of CK-MM and small fractions of CK-BB and MCK-1 isoenzymes were found. Chromosomal analysis showed that the modal chromosome number was consistently near triploid with structural abnormalities mostly involving chromosomes 1, 3 and 8, and additional unidentified markers. No alteration of chromosome 2 was observed. The RMS-GR cell line may provide a system to identify genes which are involved in the pathogenic mechanism of rhabdomyosarcomas, and to investigate the modulation of myogenic differentiation.

Key words: Rhabdomyosarcoma — Intermediate filaments — Actin — Myoglobin — Creatin kinase

Rhabdomyosarcoma is the most common soft tissue sarcoma in children, representing 4 to 8% of all pediatric malignant tumors.[1)] Although histologically three varieties are distinguishable: embryonic (including botryoid), alveolar and pleomorphic, these tumors have also been classified on the basis of their degree of differentiation.[2, 3)] Rhabdomyosarcomas are tumors of the skeletal muscle that rarely demonstrate conclusive evidence of myogenic differentiation characteristic of mature myotubes.[1)] In fact, most rhabdomyosarcomas are poorly differentiated and hence very difficult to diagnose. The diagnosis of this type of tumor involves the use of electron microscopy, conventional histopathology, and currently immunohistochemical techniques.[4)]

Desmin is the intermediate filament protein most frequently used as a marker of muscle cell differentiation because its expression is very weak in myoblasts, and increases in multinuclear myotubes resulting from the fusion of myoblasts.[5)] This protein has been used as a specific marker for rhabdomyosarcoma.[6)] However, skeletal muscle actin is also a reliable marker in the differential diagnosis of rhabdomyosarcomas, even in desmin-negative tumors.[7)] Furthermore, a combination of immunohistochemical staining using antimyoglobin and anti-CK mAbs is a useful marker in the diagnosis of childhood rhabdomyosarcomas.[4, 8)] CK exists in three forms (isoenzymes): CK-BB, CK-MB, and CK-MM.[9, 10)] Isoform CK-BB is found mainly in the brain, prostate, intestine, lung, bladder, uterus, placenta and thyroid; CK-MB is present in cardiac muscle, and CK-MM has been found mainly in skeletal and cardiac muscle. Fernández *et al*.[11)] reported different patterns of CK-BB, -MM and -MB expression in rhabdomyosarcoma cells.

The pathogenesis of rhabdomyosarcoma is unknown, but some chromosomal alterations have been implicated in the development of this tumor.[12)] Several investigators found a specific translocation t(2;13), with the breakpoint close to the Rb-1 locus in some rhabdomyosarcomas.[13)] Furthermore, frequent alterations of chromosome 3p14-21 were found in rhabdomyosarcomas by Trent *et al*.[14)]

We report the establishment and characterization of a human rhabdomyosarcoma cell line, named RMS-GR, which provides an *in vitro* system for the study of the pathogenetic mechanism of rhabdomyosarcoma and the modulation of myogenic differentiation.

[5] To whom correspondence should be addressed.

[6] Abbreviations: CK, creatine kinase; mAb, antibody; FBS, fetal bovine serum; PBS, phosphate-buffered saline; FACScan, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; APAAP, alkaline phosphatase anti-alkaline phosphatase; PAP, peroxidase-antiperoxidase.

MATERIALS AND METHODS

Case report The cell line RMS-GR was established from an embryonal rhabdomyosarcoma tumor biopsied in a 79-year-old man. The patient developed a large soft tissue mass in the urogenital tract. The resected specimen was histopathologically classified as embryonal rhabdomyosarcoma. The patient was treated with chemotherapy (doxorubicin, vincristine, cyclophosphamide and dactinomycin) after incomplete resection of the primary tumor. The patient had repeated relapses and died 8 months later despite multichemotherapy.

Cytochemical and immunohistochemical staining Tumor tissue obtained from the primary lesion was stained with hematoxylin-eosin. Immunocytochemical staining was performed with a standard APAAP technique[15] using primary mAbs (Sigma, St. Louis, MO) anti-vimentin (1:50), (clone VIM-13.2), anti-desmin (1:100) (clone DE-U-10), anti-myoglobin (1:200) (clone M61) and anti-pan-cytokeratin (1:100) (clone C-11), and the PAP method[15] was used for immunocytochemical staining with mAb anti-α-sarcomeric actin (1:100) (clone 5C5) (Sigma). Incubation omitting the primary mAb was used as a control.

Establishment of the RMS-GR cell line The intraoperative biopsy specimen of embryonal rhabdomyosarcoma was washed in fresh medium and minced into small fragments about 2×2×2 mm in size. The culture medium used was Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% FBS (Gibco), 20 m*M* L-glutamine, 3.5 mg/μl sodium bicarbonate, 4.5 g/liter glucose, 250 U/ml ampicillin and 20 μg/ml streptomycin. Cells were maintained at 37°C in an atmosphere containing 5% CO_2. Confluently cultured cells were passaged by treatment with 0.25% trypsin in PBS. The RMS-GR cells in medium containing 10% FBS and 10% dimethyl sulfoxide were sampled at intervals and stored in liquid nitrogen.

Determination of cell growth and tumorigenicity assay To determine cell growth, 2×10^5 cells were seeded onto 35-mm culture dishes and the average number of cells in triplicate dishes was counted at intervals. Cells were harvested by trypsinization and counted in a model ZBI Coulter Counter. The doubling time was obtained from the growth curve of the RMS-GR cell line by determining the time necessary for the cell population in the logarithmic phase to double. RMS-GR cells (2×10^6) were injected subcutaneously into BALB/c nude mice to assay tumorigenicity. The mice were observed for 6 weeks to follow the development of the tumor mass.

Morphologic study Cultured RMS-GR cells were examined by inverted light microscopy and were processed for electron microscopic study. The cells were fixed *in situ* with 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer (pH 7.4) for 1 h at room temperature. The monolayer was postfixed with 1% osmium tetroxide in 0.1 *M* cacodylate buffer for 1 h at room temperature, dehydrated in ethanol and then detached from the culture vessel by rapid treatment in propylene oxide and embedded in Epon 812. After polymerization, the plastic was removed and ultrathin sections were cut parallel and perpendicular to the surface of the flask. The ultrathin sections were stained with uranyl acetate-lead citrate and examined in a Hitachi H 7000 transmission electron microscope (Hitachi, Ltd., Tokyo).

Preparation of cells for FACScan Briefly, 10^6 cells were transferred to universal screw cap tubes containing sterile PBS, then washed and centrifuged at 225*g* for 5 min, discarding the supernatant. The washing and centrifugation steps were repeated once or twice. The cells were permeabilized with methanol for 10 min, then washed three times in PBS and once in distilled water. The cells were incubated for 30 min at 4°C with the appropriate mAbs. The cells were then washed twice in cold PBS and reincubated with FITC-conjugated antimouse IgG (1:50) for FACScan analysis (Becton Dickinson, Mountain View, CA). The following mAbs (Sigma) were used: anti-desmin (dilution 1:100), anti-vimentin (dilution 1:100), anti-human myoglobin (1:200), anti-α-sarcomeric actin (1:200) and anti-cytokeratin (1:100).

Electrophoretic analysis of CK isoenzymes A kit specifically designed for CK isoenzymes (Paragon Electrophoretic System, Beckman, Brea, CA) was used according to the manufacturer's instructions (Beckman Instructions 015-556461-L), and the results were evaluated visually under an ultraviolet light source, or scanned with a suitable fluorescence densitometer (Appraise, Beckman).

Chromosome analysis RMS-GR cells in the logarithmic growth phase were incubated with colchicine (Colcemid, Gibco) at a final concentration of 0.016 μg/ml of medium 2 h before harvesting with trypsin. The cells were treated with 0.05 *M* potassium chloride for 20 min at room temperature and fixed with methanol:acetic acid (3:1). The chromosome preparations were aged in an incubator at 50°C for 12 to 18 h and then incubated for 15 min at 75°C. Preparations were stained by using a modification of the trypsin-Giemsa banding method,[16] and G-banded metaphases were photographed and analyzed.

RESULTS

Primary tumor Light microscopic analysis showed that the primary tumor was composed of a diffuse growth of round or polygonal cells with sparse cytoplasm. The cells had an eccentrically located nucleus with one or more prominent nucleoli. Mitotic figures were frequently observed. Immunohistochemically, the primary tumor showed intense positive staining for vimentin (Fig. 1A),

positive staining for α-sarcomeric actin and myoglobin (Fig. 1B), and only focal staining for desmin (Fig. 1C). Cytokeratin protein was negative (not shown). The resected specimen was classified as an embryonal rhabdomyosarcoma.

***In vitro* morphology and growth of the RMS-GR cell line** The RMS-GR cell line was established from a primary tumor as described in "Materials and Methods." Light microscopic observations showed that most cells were attached to the bottom of the flasks and grew as irregular monolayers, forming confluent aggregates. Although several types of cells were seen, small spindle-shaped and rounded or polygonal cells predominated. Both types were characterized by a single nucleus, sparse cytoplasm, and considerable mitotic activity. Occasional elongated cells with longitudinal polarity were also present (Fig. 2A). Relatively few cells contained more than one nucleus, and there was no evidence of cellular fusion or the formation of multinucleated cells.

In electron microscopic images, the RMS-GR line was characterized by large, irregular nuclei (Fig. 2B). The cytoplasm contained few lipid structures and moderate amounts of rough endoplasmic reticulum and polyribosomes, with a few elongated mitochondria. Typical cytoplasmic inclusions of aggregated intermediate filaments were seen near the nucleus (Fig. 2C). Myofibril-like thin filaments were found in RMS-GR, but not dense Z-band like material (Fig. 2D). Cultures of the cells at 10th and 50th passage showed similar *in vitro* morphology without significant changes.

Under standard culture conditions, cells grew exponentially and reached saturation density, as shown in Fig. 2A. The growth rate became stable at the 10th passage. The doubling time was about 42 h at the 10th and 50th passages. Cultured cells at the 10th and 50th passages were transplanted into nude mice as described in "Materials and Methods." RMS-GR cells produced an early tumor nodule (5 mm in diameter) on the 10th day. This tumor grew rapidly, reaching 3 cm in diameter about 25 days after inoculation (Fig. 3). Morphological study of the subcutaneous tumor showed it to be similar in appearance to the primary tumor removed from the patient (not shown).

FACScan analysis of protein expression Flow cytometric analyses of the RMS-GR cell line at the 10th passage showed that 56.6% of the cells were positive for vimentin, whereas only 28.9% of the cells were positive for desmin. Myoglobin and α-sarcomeric actin were expressed on 51% and 41% of the RMS-GR cells, respectively. However, these cells were negative for cytokeratin (Table I). No significant changes were found in the RMS-GR cell line at the 50th passage.

Creatine kinase isoenzyme analysis Line RMS-GR produced a large fraction of CK-MM (64.5%) and a small fraction of CK-BB (14.9%). The fractions corresponding to CK-MB and MCK-1 represented 11.1% and 9.5% respectively of the total CK content (Table I). Similar results were found at the 50th passage.

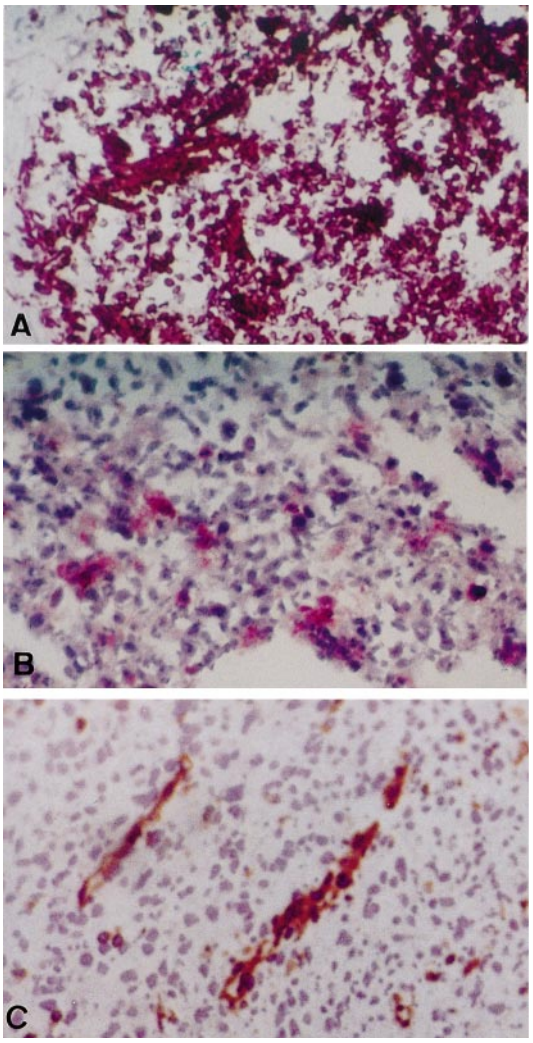

Fig. 1. Immunohistochemical staining of primary tumor sections. Intense staining with monoclonal antibody against vimentin (APAAP) (A), positive staining with monoclonal antibody against α-sarcomeric actin (APAAP) (B), and weak staining with monoclonal antibody against desmin (PAP) (C) (original magnification ×250).

Cytogenetic analysis Thirty metaphases were photographed and karyotyped. The modal chromosome number was consistently near triploid, with structural abnormalities mostly involving chromosomes 1, 3 and 8, and additional unidentified markers. A representative karyotype is shown in Fig. 4. The most interesting finding was the presence of a rearrangement involving the short arm of chromosome 8 as a common abnormality. We tentatively

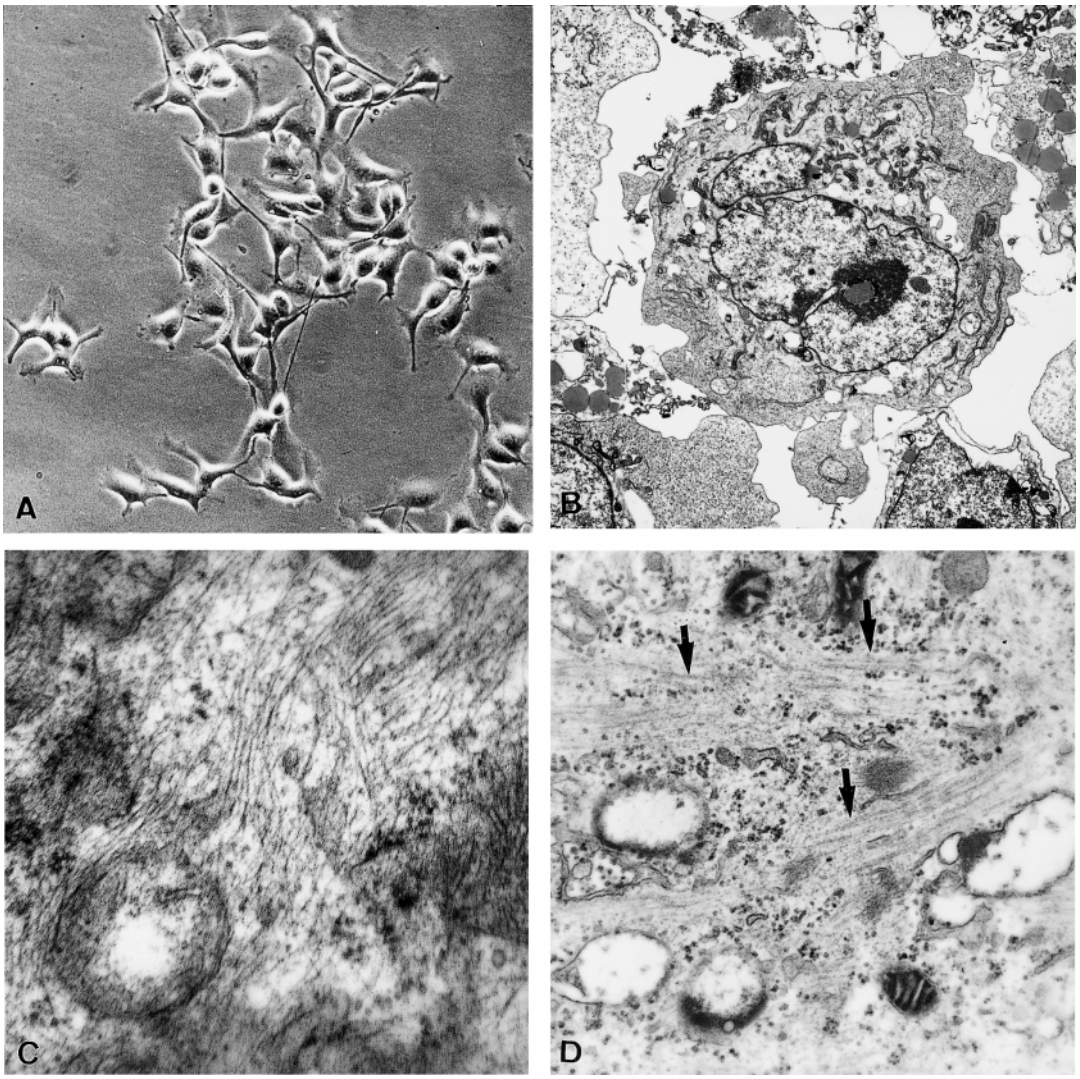

Fig. 2. Morphology of RMS-GR cells. Phase contrast microscopy of small spindle-shaped mononuclear cells under standard growth conditions. Typically shaped cells of the RMS-GR cell line formed nuclei, and grew in a monolayer (original magnification ×100) (A). Transmission electron micrograph of a polygonal cell with a large nucleus and sparse cytoplasm (original magnification ×3000) (B), showing intermediate filaments in the cytoplasm (original magnification ×43000) (C) and parallel myofilaments (original magnification ×31500) (D).

Table I. Analysis of Protein Expression (by FACScan) and CK Isoenzymes (by Electrophoresis) in the RMS-GR Cell Line

Proteins	(%)	CK isoenzymes	(%)
Vimentin	56.6±3.5	CK-MM	64.5±4.4
Desmin	28.9±2.6	CK-BB	14.9±3.8
α-Sarcomeric actin	41.0±3.8	CK-MB	11.1±5.5
Myoglobin	51.0±1.9	MCK-1	9.5±4.3
Cytokeratin	4.0±2.1		

All data are means±SEM of four separate experiments.

located the breakpoint of this rearrangement in 8p23, though the origin of the extra material could not be determined. Structural aberrations involving chromosome 1 were also found. One or two copies of a derivative del(1)(p13) were present. In addition, deletion of the short arm of chromosome 3 at p13 was observed. An imbalanced translocation probably involving the short arm of chromosome 19 and the long arm of chromosome 3 at q11 was also observed. Monosomies of chromosomes 4, 14 and 18 represented random losses. The karyotype formula was 59,XY,1,+der(1)t(1;?)(q31;?),+del(1)(p13),

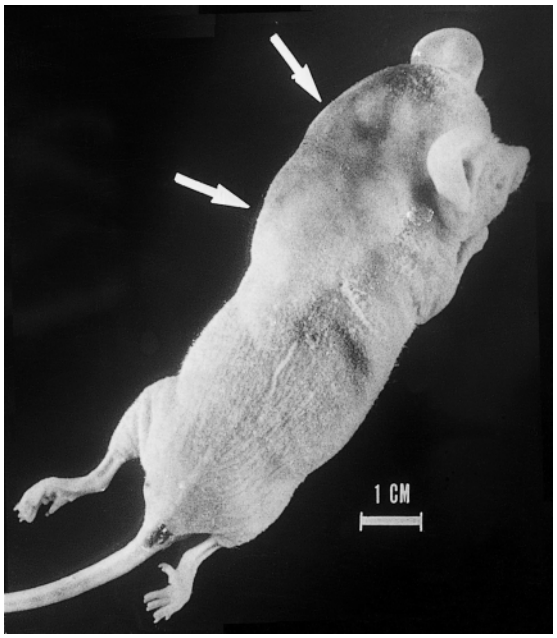

Fig. 3. Tumorigenicity in nude mice. Nude mice 30 days after subcutaneous injection of 2×10^6 RMS-GR cells.

+del(3)(p13),+der(3)t(3;19)(p11;q11),+der(8)t(8;?)(p23;?), +M. Unfortunately, it was not possible to perform chromosomal analyses of the primary tumor.

DISCUSSION

Rhabdomyosarcomas are highly malignant tumors of the skeletal muscle which usually show little or no morphological evidence of myogenic differentiation. However, the mechanism by which the capacity to differentiate is inhibited is unknown. Although cell lines derived from these malignant tumors would be useful experimental models for biological investigation, only a few rhabdomyosarcoma cell lines have been established, and most of them were obtained from childhood tumors, in which anatomopathological and immunohistochemical analyses showed a low degree of differentiation.[17–21] However, some of these lines may be induced to differentiate with pharmacological or biological agents.[22–24]

We established a new human rhabdomyosarcoma cell line from a primary tumor. Analyses of this cell line, named RMS-GR, indicated that, although several types of cells were seen, small spindle-shaped and rounded or polygonal cells predominated. Similar findings were obtained in other rhabdomyosarcoma cell lines[19, 21, 25] which showed no morphological features of differentiation under a light microscope. Our cell line did not show the spontaneous cell fusion observed in the line established by Petkovi *et al.*,[26] and in contrast with rhabdomyosarcoma cell line TS-RM-1,[21] ultrastructural analysis of RMS-GR cells showed few lipid droplets and glycogen accumulations, features considered by Enzinger and Weiss[1] as signs of differentiation. However, aggregated intermediate filaments near the nucleus and myofibril-like thin filaments were found, as in other rhabdomyosarcoma cell lines derived from adults.[27]

Because desmin and vimentin display opposite patterns of expression during development in cells of myogenic lineage,[5] these proteins have been used as classical markers of differentiation in rhabdomyosarcoma cells.[17, 18] We recently showed that vimentin and desmin expression was modified in rhabdomyosarcoma lines RD, A-673 and A-204 induced with differentiating agents, although the pattern of desmin and vimentin expression in these lines indicated a low degree of differentiation.[27] In our RMS-GR cell line the expression of these intermediate filament proteins, together with myoglobin and α-sarcomeric actin expression, strongly suggested a moderate degree of myogenic differentiation.

These results are supported by the results of analysis of CK isoenzymes. Throughout normal myogenesis, CK-MM and CK-MB isoenzymes increase steadily, displacing the CK-BB fraction more typical of embryonic tissues.[28] According to Tsokos *et al.*,[29] the presence of CK-BB together with myosin, myoglobin and/or CK-MM markers in a tumor is strongly suggestive of rhabdomyosarcoma. The CK-MM isoenzyme is a useful marker for distinguishing poorly or moderately differentiated rhabdomyosarcoma from other types of small round cell tumors in children.[30] Line RMS-GR showed a small fraction of CK-BB and a large fraction of CK-MM. We recently proposed that the pattern of CK isoenzymes is a good marker of the degree of differentiation in rhabdomyosarcomas, and found that undifferentiated rhabdomyosarcoma cells showed a CK isoenzymatic pattern characterized by high CK-BB expression. The addition of a differentiating agent such as dimethyl sulfoxide to the culture medium produced a variable increase in CK-MM and/or CK-MB isoenzymes in these tumors cells.[31] The enzymatic pattern of the RMS-GR cell line suggested a moderate degree of myogenic differentiation in contrast with other rhabdomyosarcoma cell lines, such as TE.32.7, RD and A-204[27] and the cell line established by Garvin *et al.*,[17] in which CK-MM isoenzyme expression was low.

Chromosomal analysis of rhabdomyosarcoma tumors revealed differences between alveolar and embryonal subtypes.[12] A frequent nonrandom reciprocal translocation between chromosome 2q35 and chromosome 13q14 identifed in alveolar rhabdomyosarcoma[32] is associated with a rearrangement of the PAX-3 gene, which encodes a paired box transcription factor.[33] This specific translocation has

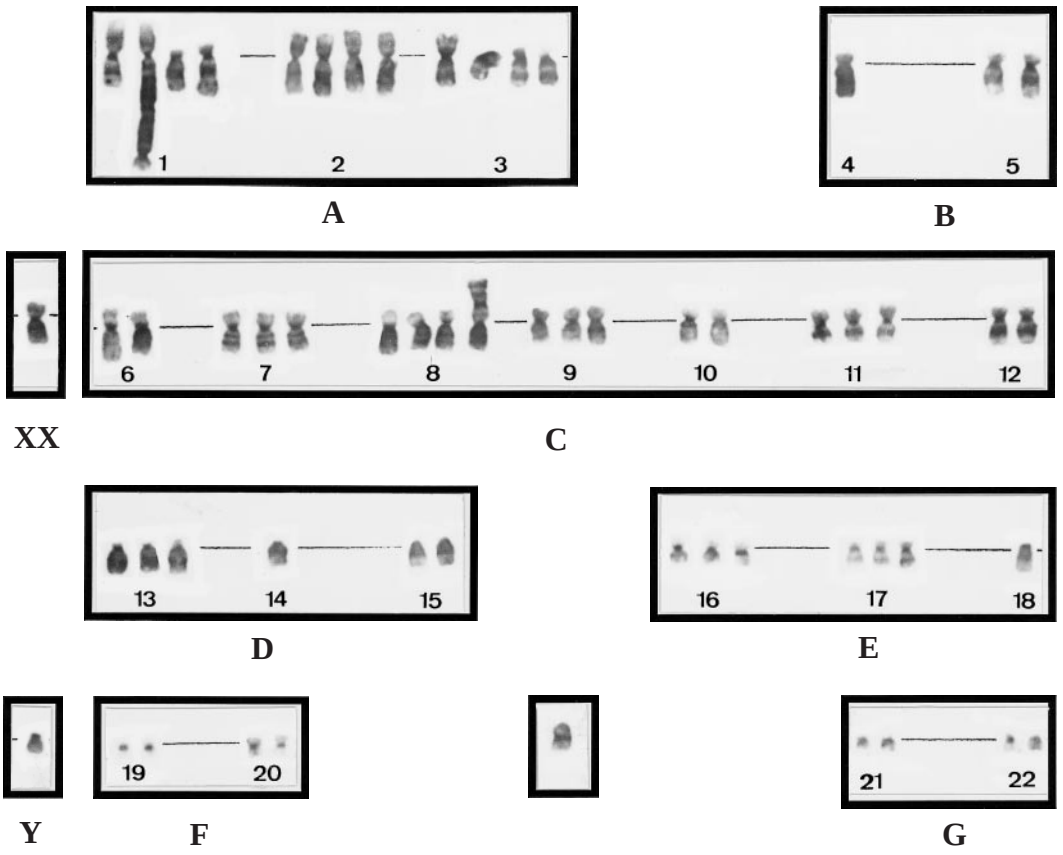

Fig. 4. A representative karyotype of RMS-GR cells: 59,XY,-1,+der(1)t(1;?)(q31;?),+del(1)(p13),+del(3)(p13),+der(3)t(3;19)(p11;q11),+der(8)t(8;?)(p23;?),+M.

also been found in the NSR-1 line, a new human rhabdomyosarcoma cell line established by Ogose *et al.*[34] However, this translocation is not present in embryonal rhabdomyosarcoma.[21, 35] This abnormality was not found in RMS-GR cells. The most interesting cytogenetic features of our cell line were structural abnormalities involving chromosomes 1, 3 and 8, and additional unidentified markers. Monosomies of chromosomes 4, 14 and 18 were random losses, and no alteration in chromosome 2 was observed. Although no specific chromosomal alteration has been found in embryonal rhabdomyosarcoma, an abnormality in chromosome 1 is apparently a frequent finding in childhood malignancies.[36] Similar results have been found in the cell line established by Magnani *et al.*[37]

In conclusion, we have established a new rhabdomyosarcoma cell line, termed RMS-GR, which may provide an experimental system to study the pathogenic mechanism of rhabdomyosarcomas and to investigate the myogenic differentiation of neoplastic and normal muscle cells. This new cell line, in contrast with most other rhabdomyosarcoma cell lines, was obtained from an adult tumor, and shows a moderate rather than a low degree of myogenic differentiation. Further research is in progress to identify the genes that may be involved in the morphological, biochemical and cytogenetic alterations observed in this cell line.

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