Molecular cytogenetic characterization of rhabdomyosarcoma cell lines

Sandra Rodriguez-Perales, Angel Martínez-Ramírez, Sara Alvarez de Andrés, Laura Valle, Miguel Urioste, Javier Benítez, Juan C. Cigudosa*

Cytogenetics Unit, Department of Human Genetics, Centro Nacional de Investigaciones Oncológicas, C/ Melchor Fernandez Almagro 3, 28029 Madrid, Spain

Abstract

Alveolar rhabdomyosarcomas (ARMS) are soft-tissue tumors that are genetically characterized by the presence of reciprocal translocations that generate the fusion gene *PAX3-FOXO1A* or *PAX7-FOXO1A*. For the study of the biologic consequences of such rearrangements, several cell lines have been generated. However, established cell lines accumulate chromosome and genetic aberrations that make it difficult to draw significant conclusions. We have applied a set of techniques that includes spectral karyotyping, fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), and microarray CGH, to the most commonly used cell lines carrying the two fusion genes that are present in ARMS. We have identified the bacterial artificial chromosomes that cover the breakpoints at genes *PAX3*, *PAX7*, and *FOXO1A*, which can be used as FISH probes for the translocations. The RH30 cell line, positive for the *PAX3-FOXO1A* fusion gene, was found to be highly complex: wide range of chromosome number, more than 50 chromosome rearrangements, amplification of the hybrid gene, 24 DNA changes detected by conventional CGH, and 21 gene copy changes detected by microarray CGH (including several high-level amplifications). RMZ-RC2 cell line, positive for the *PAX7-FOXO1A*, was in the near-tetraploid range with only nonclonal structural rearrangements, amplification of the hybrid gene, 24 DNA changes by CGH, and 8 gene copy changes, confirming the previously reported high-level amplification of *MYCN*. © 2004 Elsevier Inc. All rights reserved.

1. Introduction

Soft-tissue tumors represent a heterogeneous group of mesenchymal lesions, and their classification is the subject of continuous debate [1]. Rhabdomyosarcomas (RMSs) are malignant mesenchymal tumors [2,3]. They represent the most common soft-tissue sarcoma of childhood. They are histologically classified as embryonal (ERMS), the most commonly occurring variant, and alveolar (ARMS). Diagnostic differentiation of these variants is important. The embryonal variant has a relatively good prognosis, whereas alveolar RMS is a highly malignant tumor. Rhabdomyosarcomas are poorly differentiated by light microscopy. Chromosome analysis, molecular cytogenetics, and molecular assays may become increasingly useful in the diagnosis, characterization, and classification of soft-tissue tumors.

In almost all ARMS tumors, the specific t(2;13)(q35;q14) or its variant, t(1;13)(p36;q14), has been identified by cytogenetic or molecular analysis. The molecular consequences of these translocations have been fully characterized and they correspond with the fusion of the *PAX3-FOXO1A* genes in the t(2;13) [4] and of the *PAX7-FOXO1A* genes in the t(1;13) [5]. In some alveolar RMS, the fusion genes are amplified. These findings indicate that translocation and amplification can occur sequentially in a cancer to alter both the structure and copy number of a gene and thereby activate oncogenic activity by complementary mechanisms [6]. Whereas consistent chromosome translocations characteristic of ARMS have been reported, no such specific cytogenetic abnormality has yet been described in ERMS [7].

Transformed cell lines are widely used as models of cancer and represent an excellent resource for genomic studies. There are many cell lines from RMS, and most of them contain the t(2;13) translocation. The RH-30 cell line has become one of the most frequently used cell lines. This cell line was established from the bone marrow metastasis of a 17-year-old man with undifferentiated alveolar rhabdomyosarcoma; cells were described as carrying a p53 mutation, expressing high levels of both myogenin and myoD, and expressing

the fusion protein secondary to t(2;13) [8]. The only known cell line that harbors the variant t(1;13) is RMZ-RC2. This cell line is a subclone of the RMZ line, which was derived from a bone marrow metastasis of a 2-year-old child with ARMS. RC2 cells show amplification of the *PAX7-FOXO1A* fusion gene and of the *MYCN* oncogene, both expressed cytogenetically as double-minute chromosomes [9].

These cell lines have not been studied in detail cytogenetically. The conventional cytogenetic analysis revealed complex karyotypes with several markers. For this reason, we used molecular cytogenetic techniques: spectral karyotyping (SKY), comparative genomic hybridization (CGH), comparative genomic hybridization array (CGH array), and gene-specific probes to better characterize the chromosome abnormalities of the two cell lines. The characterization of the cell lines would enhance their usefulness in studies for clarifying the knowledge of the biology of RMS.

2. Materials and methods

2.1. Cell culture and metaphase preparation

The cell line RH-30 was acquired from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and cultured in RPMI 1640 containing 10% fetal calf serum (FCS), 45 IU/mL penicillin, and 45 μg/mL streptomycin. The RMZ-RC2 cell line, provided by Dr. P.L. Lollini (Cancer Institute, University of Bologna, Bologna, Italy), was grown in Dulbecco's modified Eagle medium fortified with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Both cell lines were incubated at 37°C in culture flasks in an atmosphere of 5% CO_2 in air. Metaphase cells were prepared by standard cytogenetic methods. Mitotic arrest with Colcemid (0.1 μg/mL, 1.5 hours, 37°C; GIBCO, Strachclyde, UK) was followed by hypotonic treatment (75 mM KCl, 15 minutes, 37°C) and fixation with methanol/acetic acid (3:1) before spreading onto slides.

2.2. Fluorescence in situ hybridization analysis (FISH)

Two sets of probes were used to detect t(2;13) and t(1;13). We used bacterial artificial chromosomes (BAC) that cover the region of the genes *PAX3*, *PAX7*, and *FOXO1A* as hybridization probes. The BAC were obtained from Human BAC Clone Library RPCI-11 (Children's Hospital Oakland Research Institute, Oakland, CA). The following BAC were used: 394P21 for the *PAX7* gene, 384O8 for the *PAX3* gene, and 487O12 for the *FOXO1A* gene. The BAC that cover each gene were labeled with different colors by nick translation (Vysis, Downers Grove, IL). The BAC were used to detect the *PAX3-FOXO1A* and *PAX7-FOXO1A* rearrangements. Commercial gene-specific probes were also used for cyclin D1 and *MYCN* genes (Vysis). Cell images were captured using a cooled charge-coupled device (CCD) camera (Photometrics SenSys camera) connected to a computer running the Chromofluor image analysis system (Cytovision; Applied Imaging Ltd., New Castle, UK).

2.3. CGH

CGH analysis was performed as described previously [10]. RH-30 and RC2 cell lines DNA (test) and normal DNA were differentially labeled by CGH–nick translation (Vysis) with SpectrumGreen dUTP and SpectrumRed dUTP, respectively. The CGH metaphases were captured through a fluorescent microscope (Olympus BX60) and a CCD camera (Photometrics Sensys camera), and then analyzed using the chromofluor image analysis system (Cytovision; Applied Imaging Ltd.). The green and red fluorescence intensities were calculated, and the green/red ratio profiles along the chromosome axis were displayed. Ratios greater than 1.2 and less than 0.8 represented chromosomal gains and losses, respectively. Telomeric and heterochromatic regions were excluded from the analysis.

2.4. CGH array

We labeled 500 ng of cell line DNA (test) and 500 ng of genomic DNA (reference) with Alexa-488 (green) and Alexa-594 (red) (Molecular Probes, Eugene, OR, USA), respectively. After that, the labeled DNA was purified using Qiaquick PCR purification columns (Qiagen, West Sussex, UK) and resuspended in 25 μL hybridization buffer (Vysis). The hybridization solution was heated at 80°C for 10 minutes, incubated in the dark for 2 hours, and then hybridized to the AmpliOncTM I DNA array (Vysis) for 24 hours. This array is composed of 58 different genomic DNA fragments. These clones have been reported to be associated with tumor formation through amplification. After a series of washes, the chip was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The hybridized array was analyzed with the GenoSensor Reader System (Vysis). The test/reference ratios are defined as the ratio of the sum of test intensity pixel values to the sum of the reference intensity pixel values. Each clone was spotted in triplicate so that the total value for each clone was calculated as the mean value of the individual ratios. The Pearson's r correlation of a scatterplot of test versus reference signal intensities for the pixels in each target was calculated. Data from targets with $r < 0.8$ were discarded. The most appropriate fluorescence threshold for gains of gene copy number was set to be greater than 1.25, and that for losses of gene copy number was set to be less than 0.75. To exclude false-positives and false-negatives, appropriate controls were used.

2.5. SKY

Before hybridization, the chromosome spreads were treated with pepsin (0.001% pepsin in 0.01 N HCl, 37°C). The human SKY probe mixture (Applied Spectral Imaging, Migdal Ha-Emeck, Israel) was applied to the preparations and hybridized for 48 hours. Washing of preparations and

detection of probe was done according to the instructions of the manufacturer. The chromosome spreads were counter-stained with DAPI. Image acquisition was performed with the SpectraCube system (Applied Spectral Imaging, Migdal-Ha-Emeck, Israel) mounted on an Axioplan 2 microscope (Zeiss, Jena, Germany), and SKY View imaging software was used for metaphase analysis.

3. Results

A comprehensive cytogenetic/molecular characterization of two rhabdomyosarcoma cell lines, RH30 and RMZ-RC2, was performed using G-banding, SKY, CGH, and microarray CGH. The combined use of all techniques allowed us to obtain an enhanced description of the structural aberrations, which included balanced and unbalanced translocations, deletions, and insertions. The complete description of rearrangements is given in Table 1. The chromosome rearrangements were more complex in RH30 than in RMZ-RC2 (Fig.1). Conventional and microarray CGH showed a high number of DNA copy changes in both cell lines.

3.1. G-banding

The karyotype of RH30 has not been published. The chromosome number of RH30 revealed a main population of cells in the near-triploid range (with a broad variation in the number between 51 and 87 chromosomes). The image of the karyotype with QFQ bands of RMZ-RC2 has been published [9]. These authors, however, only described the chromosome number of the line and a possible t(2;10)(?p16;?q22). Our clone from the RMZ-RC2 cell line was homogenous in chromosome number, with a near-tetraploid range (between 81 and 86 chromosomes) but with a high number of double minutes and frequent monosomies of chromosomes 1, 3, 5, 6, 8, 9, 10, and 19.

3.2. SKY analysis

By SKY analysis of the RH30 cell line, we detected 52 structural alterations. Among these, 39 were found recurrently in almost all metaphases and they are described in Table 1. Some of them were very complex rearrangements, such as der(2)t(2;13;14;18)(p21;q14;?;?). The majority of the markers could not have been identified by conventional banding techniques. Translocations affecting bands 2q35 and 13q14, the chromosome sites of *PAX3* and *FOXO1A*, were noted although the rearrangements were unbalanced and complex (Fig. 1). The study of the RC2 cells revealed 14 structural and 38 numerical alterations. The detailed description of the structural abnormalities is shown in Table 1. Compared with the previously published findings on this cell line, we also found some telomeric fusions affecting distinct chromosomes as well as a varying number of double minutes. The SKY analysis showed no rearrangements involving the *PAX7* or *FOXO1A* gene sites at bands 1p36 or 13q14, respectively (Fig. 1).

3.3. FISH analysis

PAX3-, *PAX7*-, and *FOXO1A*-specific probes were designed by constructing contigs covering their respective genomic regions, as described previously [11]. For each gene, we used a panel of three BAC covering the translocation breakpoints. The probes were hybridized to metaphase spreads and interphase nuclei to determine if these genes were altered in the two cell lines. In the RH30 cell line, we observed nonrearranged signals of the genes *PAX3*, *PAX7*, and *FOXO1A*. In addition, we observed four fusion signals of the *PAX3-FHKR* fusion gene, inserted at different chromosome points. Moreover, the fusion gene *PAX3-FOXO1A* was amplified in the form of a homogeneously stained region (HSR) and inserted in several derivative chromosomes (Fig. 1). We did not find any fusion gene for the *PAX7-FOXO1A* chimeric gene. In the RC2 cells we noticed multiple copies of the *PAX7-FOXO1A* chimeric gene. The fusion signals were localized exclusively on some double minutes. We also performed cohybridization FISH with the commercial *MYCN* probe and our probes (data not shown). We observed that multiple signals of *MYCN* were located on some of the double minutes and that the double minutes that showed a signal of the *PAX7-FOXO1A* fusion gene were different from those showing signals from the *MYCN* probe. We did not observe signal fusions for the *PAX3-FOXO1A* chimeric gene.

Table 1
Chromosome structural rearrangements detected by SKY and G-banding in RH-30 and RMZ-RC2 rhabdomyosarcoma cell lines

Cell line	Karyotype
RH-30	51~87<92>,XY,der(X)t(X;15)(?;q?),tas(Y;12)(q11.1;q11),der(1)t(1;15)(q?;q?),der(1)t(1;13;14)(p36;?;?),der(1)t(1;8)(p33;?),der(1)t(1;7)(?;?), del(2)(q13),der(2)t(2;13)(q35;q14)t(2;9)(p21;?),der(2)t(2;13;14;18)(p?;?;?;?),der(2)t(2;11;17)(?;?;?),der(4)i(4)(q10)t(4;14)(q?;q?), der(5)t(5;10)(?;?),del(6)(p11),del(6)(q13)der(6)t(6;14)(q35;q?),del(7)(q?),der(8)i(8)(q10)t(4;8;11)(?;q?;?),der(9)t(5;9)(?;?), der(9)t(9;17)(q12;q?),del(10)(q11.2),i(10)(p10),der(11)t(11;20)(p?;?),der(11)t(11;17;?)(q23;q?;?),der(12)t(3;12)(?;p13),der(12)t(6;12)(?;q13), der(13)t(4;11;13;17)(?;?;q?;?),der(14)i(14)(q10)ins(14;3)(q?;?),del(16)(q12),der(16)t(10;16)(?;?),der(18)t(2;11;18)(?;p?;q?),dic(18;20)(q?;p?), der(20)t(8;20)(?;q?),der(21)t(5;21)(?;q?),der(21)t(11;21) (q13;q13),der(22)t(3;22)(?;p13),der(22)t(4;22)(?;q?),der(22)t(1;10;22)(p?;q?;q11.2),der(22)t(6;10;22)(?;?;?)[cp10]
RMZ-RC2	81~86<92>,XXYY,−1,−3,−5,−6,−8,−9,−10,−12,−13,−19,10~25dmin[cp8] Nonclonal chromosome structural rearrangements: tas(2;4)(p;q),tas(3;22)(p;q),tas(4;7)(q;p),der(5)t(5;16)(q13;q13),der(8)t(7;8)(?;q),der(8)t(2;8)(?;?),tas(8;13)(q;q),r(10),tas(Y;10)(q;q),der(10) t(10;17)(?;?),der(10)t(4;10)(?;?),der(13)t(1;13)(p10;p10),der(14)t(3;14)(q13.1;p11.13),der(16)t(5;16)(?;)

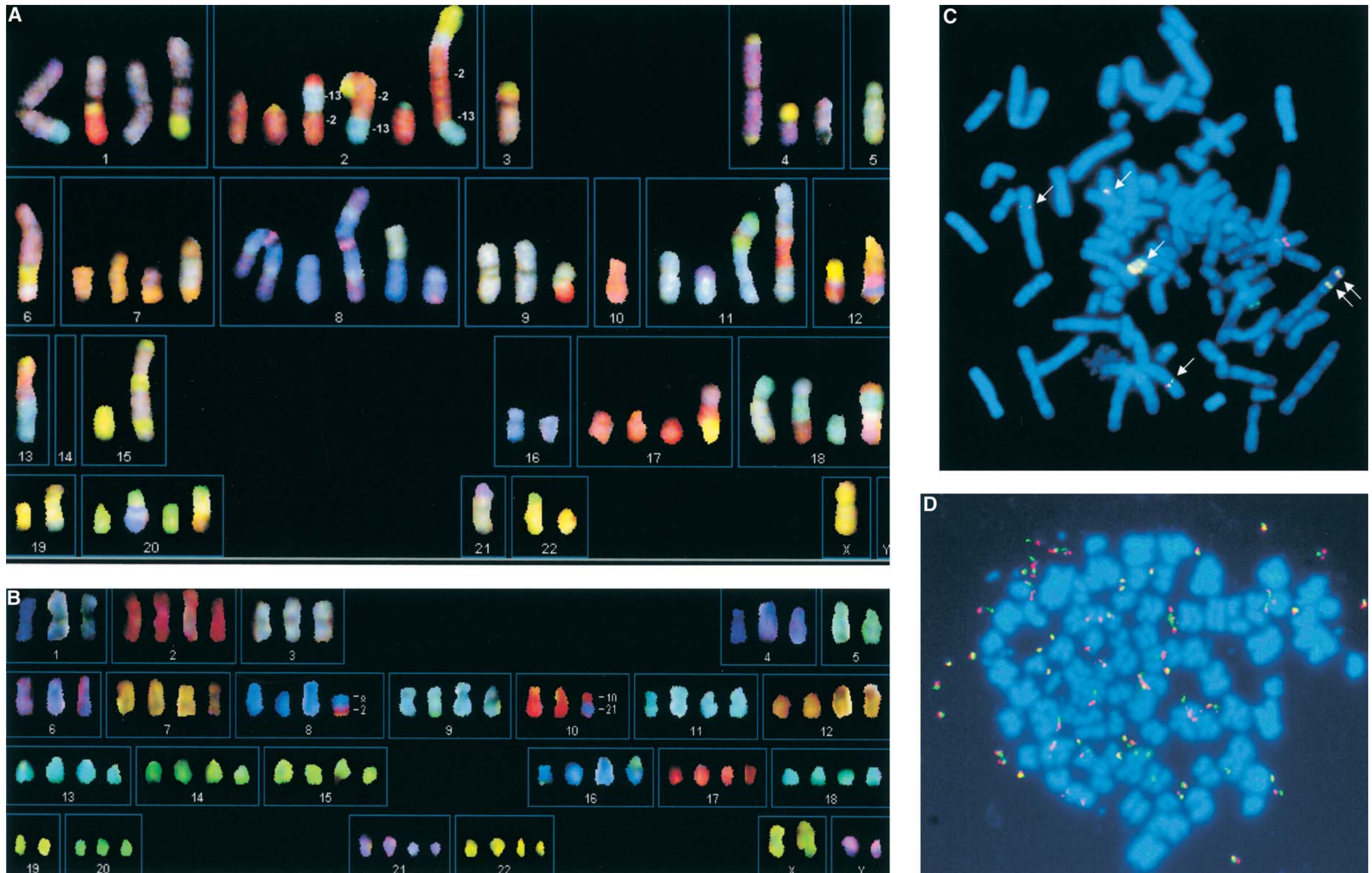

Fig. 1. SKY and FISH analyses of the cell lines. (*A*) Spectral karyotype of a representative metaphase cell of the RH-30 rhabdomyosarcoma cell line. Chromosomes affected by the t(2:13) are indicated with their respective numbers. (*B*) Spectral karyotype of a cell of the RMZ-RC2 cell line. (*C*) FISH on a metaphase of the RH-30 cell line. Five yellow fusion signals, and one region of fusion signal amplification, all generated by the t(2;13), are indicated with arrows. The green and red signals correspond to the normal *FOXO1A* and *PAX3* genes, respectively. (*D*) FISH image of a representative metaphase of RMZ-RC2 cell line. The fusion signal, generated by the t(1;13), is localized in the double-minute chromosomes.

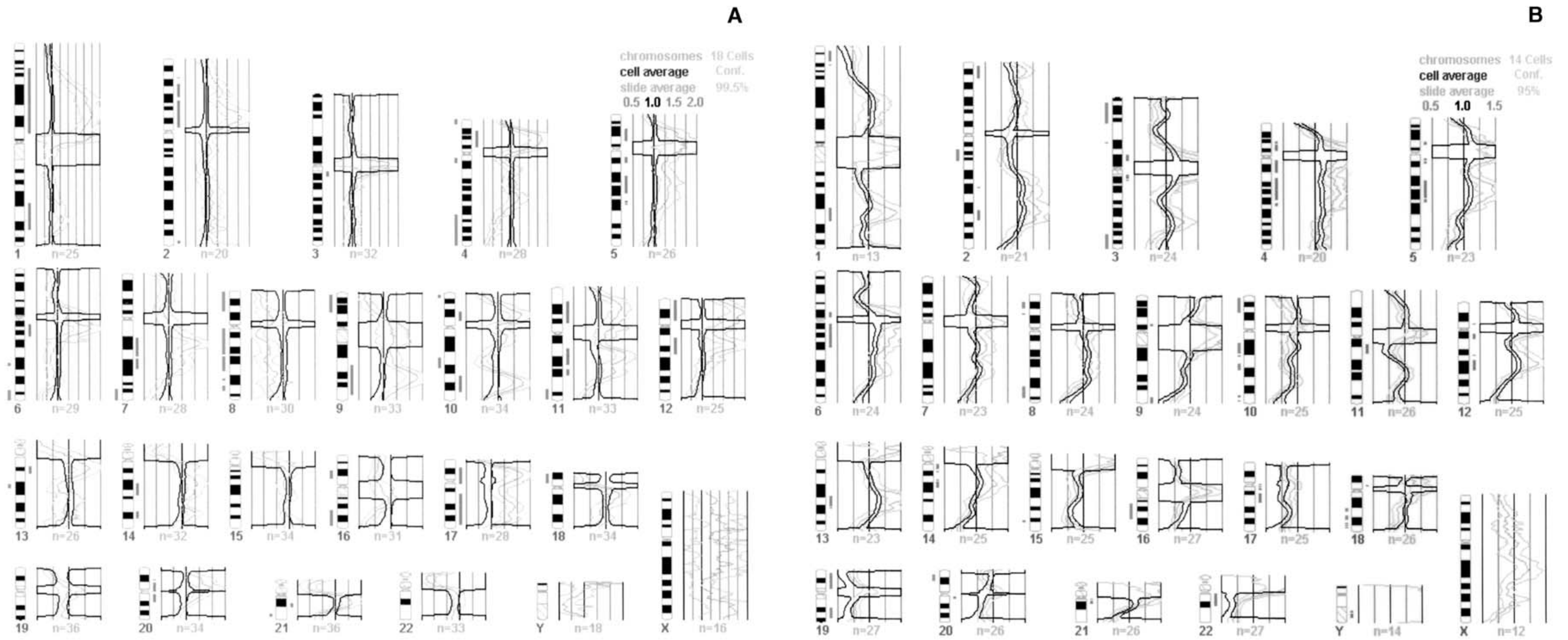

Fig. 2. CGH profiles of the two rhabdomyosarcoma cell lines. Gray bars to the right of each chromosome represent DNA copy gains and gray bars to the left of each chromosome represent DNA losses. Standard reference intervals are in black. (*A*) RH-30 cell line. (*B*) RMZ-RC2 cell line.

3.4. CGH analysis

The cell lines showed multiple aberrations, including both gains and losses of chromosomal material. The study of the RH30 cells showed that chromosome arm gain affected 12p, whereas regional gains affected 1p, 1q, 2p, 3q, 4p, 5p, 6q, 7q, 9q, 10q, 11p, 11q, 12q, 17p, and 17q. Chromosome losses affected 4p, 4q, 6q, 9p, 16q, 18p, 19p, and 21q. High-level gains were noted at the following six sites: 1p13~p32, 2p12~p14, 4p13~p15, 6q13~q14, 12p11~p13, and 12q13.3~q14. The RC2 cells had regional gains affecting 1, 2, 3, 4, 5q, 6, 11q, 12q, 13q, 14q, 17q, 19, 21q, and 22q, whereas regional losses affected 2q, 3p, 3q, 8p, 8q, 10p, 10q, 16q, 18q, and 20p. High-level gains were noted at 1q32, 5q14~q21, and 13q22 (Fig. 2).

3.5. CGH array analysis

The results of the CGH array analysis are illustrated in Table 2. Only significant values of DNA copy number changes (mean signal gain >1.25, mean signal loss <0.75) are displayed. The complete profile of the AmpliOnc microarray is showed in Fig. 3. High-level amplification signals were noted in RH30, with probes for *MET* on 7q and *GLI* and *SAS/CDK4* on 12q13. In RC2, a single point of high-level amplification affecting *MYCN* was detected. CGH array analysis was validated by FISH using *CCND1*-, *MET*-, *MYCN*-, and *PIK3CA*-specific probes.

Table 2
DNA copy changes as detected by CGH-array in RH30 and RMZ-RC2 cell lines

Name	Cytogenetic location	Mean	CV %[a]	c-CGH
RMZ-RC2				
MYCN	2p24.1	7.06	9	+
PIK3CA	3q26.3	0.59	3	+
ABL1	9q34.1	1.34	9	+
MLL	11q23	1.26	6	−
GLI	12q13.2~q13.3	0.74	7	−
RPS6KB1	17q23	1.62	9	−
BCL2 3′	18q21.3	0.63	5	+
CCNE1	19q13.1	0.73	8	−
AR 3′	Xq11~q12	0.71	6	+
RH30				
FGR	1p36.2~p36.1	1.49	3	−
NRAS	1p13.2	1.65	3	+
MYCN	2p24.1	1.60	4	−
REL	2p13~p12	1.37	2	+
PIK3CA	3q26.3	1.31	5	−
PDGFRA	4q12	0.70	6	+
EGFR	7p12.3~p12.1	0.75	1	−
PGY1	7q21.1	1.75	2	+
MET	7q31	2.87	8	+
CCND1	11q13	0.84	2	−
EMS1	11q13	1.33	5	+
GARP	11q13.5~q14	1.51	5	+
PAK1	11q13.5~q14	1.49	2	+
KRAS2	12p12.1	1.56	8	+
GLI	12q13.2~q13.3	3.51	5	+
SAS/CDK4	12q13.3	5.77	1	+
AKT1	14q32.3	1.47	2	+
FES	15q26.1	1.59	4	−
JUNB	19p13.2	1.35	1	−
AIB1	20q12	0.73	5	−
PTPN1	20q13.1~q13.2	0.74	3	−
AR5′	Xq11~q12	0.75	5	+
AR3′	Xq11~q12	0.70	4	+

Only those clones that reached significant values (gain >1.25; loss < 0.8) are displayed.

Abbreviations: CV, coefficient of variation; +, concordance with chromosome CGH changes.

[a] CV = standard deviation/mean over the included signals of the normalized per-signal test to reference ratio by means.

4. Discussion

We report here the cytogenetic characterization of two rhabdomyosarcoma cell lines by SKY, CGH, CGH array, and FISH. SKY analysis revealed complex karyotypes. These lines have accumulated numerous chromosomal structural aberrations. Many of the karyotypes from primary samples of RMS showed complex rearrangements [12–14]. Some of these rearrangements have also been detected in the RH-30 and RMZ-RC2 cell lines, together with other chromosomal abnormalities that have not been seen in primary samples [12,13]. This may reflect genetic differences between primary tumor samples and cultured cell lines.

The combination of molecular cytogenetic techniques enabled us to define the first comprehensive karyotype for RH-30. This cell line has a very complex karyotype with many numerical and structural abnormalities. Translocations affecting the *PAX3* and *FOXO1A* gene sites were noted. By the design of our own probes, we could carry out FISH analysis of the translocation status in the cell lines. The FISH analysis revealed several fusion signals of *PAX3-FOXO1A*. The characterization of the RH-30 cell line also revealed the amplification of the fusion gene. This event has been frequently described in tumors that harbor t(1;13) [14], but has only been described in one case of RMS with t(2;13) [6].

RMZ-RC2 showed a near-tetraploid karyotype with many numerical alterations and a few structural aberrations. Double minutes were detected in all the metaphases studied. The SKY study did not show the t(1;13) affecting the *PAX7* and *FOXO1A* genes. FISH analysis revealed multiple copies of the *PAX7-FOXO1A* fusion gene localized on a subset of double minutes. Signals corresponding to the *N-MYC* probe were detected in the remaining double minutes. We confirmed the cytogenetic findings reported in a previous article [9] and identified several novel marker chromosomes.

The CGH study complemented the SKY karyotype. Gene amplification is regarded to be a reflection of genetic instabilities in solid tumor cells. We found that these cell lines have several chromosome regions with gain and/or loss of genetic material. Some of the observed gains and losses are reportedly altered in RMS tumor cases [15]. The gained regions are 7q, 11q, 12q, 12q13.3~q14, and 20p, and the regions of

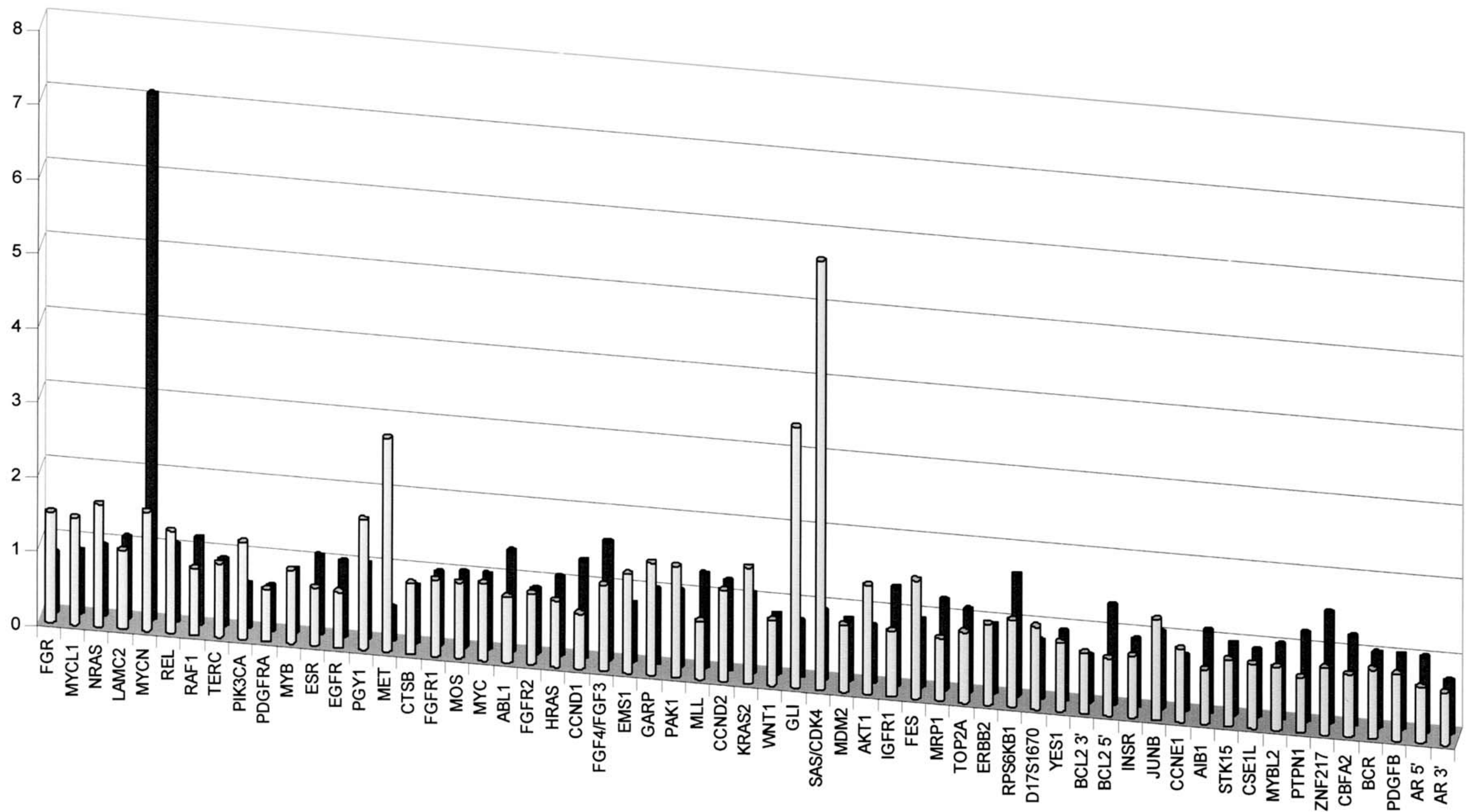

Fig. 3. Array CGH analysis of the two rhabdomyosarcoma cell lines. The complete list of the 57 analyzed genes appears on the *x* axis. The value of the test/reference ratio for a gene with no changes is between 0.75 and 1.25. Several gains and losses were observed. High-level amplifications of *NMYC*, *MET*, *GLI*, and *SAS/CDK4* are seen clearly in the graphic. Gray columns represent RH30, while those in black represent RMZ-RC2.

loss are 16q, 3p, 10q, and 16q. We have found regions that showed high-level amplifications. One of these regions, 12q13.3~q14, has been reported previously as amplified in RMS cases [15]. In addition, the majority of regions with DNA copy changes that we have observed have also been reportedly altered by CGH in different types of bone and soft-tissue sarcoma [16]. Almost all the gained regions have been previously reported, with the exception of 4p, 10q, and 22q. For the regions of loss, all those we observed have also been found altered in some soft-tissue sarcomas, with the exception of 18p, 19p, 21q, and 20p [16].

CGH array analysis allows simultaneous examination of 57 oncogenes commonly amplified in various human cancers. It detects genomic imbalances at a much higher resolution than conventional CGH (Table 2). We found concordance between the available CGH array data and conventional CGH results. To validate the array data, we performed FISH analysis with some of the genes that the CGH array showed to be altered. These genes were as follows: *CCND1*, *MET*, *MYCN*, and *PIK3CA*. We used specific probes for each gene. We selected these genes because *MYCN* and *MET* are genes found frequently amplified in RMS, and they probably play an important role in the development of RMS [17,18]. *Cyclin D1* was used as representing a deletion gene, and *PIK3CA* was chosen because it is a protooncogene that plays an important role in regulating cell growth and apoptosis but has not been found to be altered in rhabdomyosarcoma cases [19].

The FISH studies confirmed the amplification of these genes and allowed us to demonstrate that these genes were altered. Of special interest are the high-level amplifications. *MYCN* has been reportedly amplified in alveolar RMS cell lines and tumors [17]. The *MYCN* oncogene encodes a phosphoprotein that acts as a transcription factor and is involved in the regulation of cell proliferation and differentiation in normal and cancer cells. Another gene that showed a high-level amplification was *MET*. This gene encodes a receptor factor involved in growth and motility signaling. The aberrant expression of *MET* provides RMS cells with the same property as embryonal myoblasts, migrating into the surrounding connective tissues [18]. The amplicon observed in 12q13 by conventional CGH was confirmed with the microarrays. This amplicon has been found to be associated with ARMS cases. This region was covered in the array by probes that include *GLI*, *SAS/CDK4*, and *MDM2*. The use of the microarray allowed a better definition of the amplificated region that included *GLI* and *SAS/CDK4* but not *MDM2* [20]. The amplification of *GL1*, *CDK4*, and *MDM2* has been previously reported in RMS [21–23].

In summary, we have carried out a complete molecular cytogenetic characterization of two RMS cell lines, RH-30 and RMZ-RC2, that harbor t(2;13) and t(1;13), respectively. We described the BAC containing the breakpoints of *PAX3*, *PAX7*, and *FOXO1A* genes. We demonstrated that the RH-30 cell line shows amplification of the fusion gene *PAX3-FOXO1A*, whereas in RMZ-RC2, the fusion gene amplified is *PAX7-FOXO1A*. In addition, as the the CGH array revealed, there are some other gene amplifications and deletions that should be considered when these cell lines are used as biologic models of RMS.

Acknowledgments

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