Interphase Cytogenetics for the Detection of the t(11;22)(q24;q12) in Small Round Cell Tumors

Marco Giovannini,* Licia Selleri,* Jaclyn A. Biegel,‡ Katia Scotlandi,§ Beverly S. Emanuel,‡ and Glen A. Evans*

**Molecular Genetics Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037; ‡Division of Human Genetics and Molecular Biology, Children's Hospital, and Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104; and §Laboratory of Oncological Research, Instituti Ortopedici Rizzoli, Bologna 40136, Italy*

Abstract

Among the small round cell tumors differential diagnosis is particularly difficult for their undifferentiated or primitive character. In this mixed group of tumors, only the primitive neuroectodermal tumors, which include Ewing's sarcoma (ES), show the unique and consistent feature of the (11;22)(q24;q12) translocation, which can therefore be considered a hallmark of these neoplasias. We analyzed four primitive neuroectodermal tumor cell lines, one osteosarcoma cell line, and 11 patients by fluorescent in situ hybridization with cosmid clones 23.2 and 5.8, bracketing the t(11;22) at 11q24. Metaphase spreads from tumor cell lines, and from biopsy specimens of three patients with ES were analyzed. In the remaining eight patients comprising five ES, two small cell osteosarcomas and one chronic osteomyelitis, only nuclei preparations were available for analysis. We detected the t(11;22) in interphase nuclei of the four primitive neuroectodermal tumor cell lines, of three patients in which the karyotype demonstrated the translocation and in five cases of ES in which cytogenetic analysis had not been possible. Two cases of small cell osteosarcoma and one chronic osteomyelitis were also analyzed and were both normal with respect to the t(11;22). By analyzing cell lines and small round cell tumor samples by fluorescent in situ hybridization, we established that interphase cytogenetics is a rapid alternative to chromosomal analysis for the detection of the t(11;22) and represents an invaluable tool for the differential diagnosis of small round cell tumors. (*J. Clin. Invest.* 1992. 90:1911–1918.) Key words: Ewing's sarcoma • primitive neuroectodermal tumors • in situ hybridization • chromosomal translocations

Introduction

The differential diagnosis of the so-called "small round cell tumors" is particularly difficult due to their undifferentiated or primitive character (1, 2). In the last few years a variety of ancillary diagnostic techniques, such as immunohistochemistry, electron microscopy, short-term in vitro cultures, cytogenetics, and molecular genetics have provided precious tools for addressing this diagnostic dilemma (3). Furthermore, the implementation of new and more precisely tailored therapeutic protocols now requires as precise a diagnosis as possible (4, 5).

Advances in cytogenetic techniques such as analysis of tumor metaphases after direct cell harvesting and very short-term in vitro cultures, have enabled cytogeneticists to perform analysis on a wide spectrum of solid tumors (6–9). However, molecular studies of translocations in solid tumors lag far behind their study in leukemias due to the technical difficulties of chromosome analyses in fresh tissue samples (10). Furthermore, many solid tumors exhibit multiple chromosomal abnormalities which make linking consistent cytogenetic changes with malignancy extremely difficult (11, 12).

One of the exceptions is Ewing's Sarcoma (ES),[1] a malignancy of bone and soft tissues that most often affects young adolescents (13) and is associated with a t(11;22)(q24;q12) reciprocal translocation (14, 15). Cytogenetically indistinguishable translocations also occur in peripheral neuroepithelioma (PN) and Askin's tumor (16). ES and PN have indistinguishable patterns of expression of various protooncogenes (17) and it has been suggested that they represent extremes of a wide spectrum of primitive neuroectodermal tumors (PNETs), ranging from the more differentiated peripheral neuroepithelioma at one end, to the atypical Ewing's sarcoma in the middle, to the classic Ewing's sarcoma at the least differentiated end (18). The diagnosis of ES often depends on the lack of differentiated features that may be present in the other malignant small round cell tumors, rhabdomyosarcoma, neuroblastoma, non-Hodgkin's lymphoma of the bone, and small cell osteosarcoma (19). However, in this mixed group of small round cell tumors, the PNETs show the unique and consistent feature of the (11;22)(q24,q12) translocation, which has never been observed in any other small round cell tumor of bone and which can therefore be considered a hallmark of these neoplasias (14–16).

Entire genomes, chromosomal regions, and unique sequences can be specifically visualized in metaphase and in interphase cells by the use of fluorescent in situ suppression hybridization (FISSH) (20–22). Chromosomes and chromosomal abnormalities can be highlighted, and the chromosomal location of specific genes can be determined (20–22). In fact, FISSH plays a pivotal role in gene mapping, tumor biology, prenatal diagnosis, and cytogenetics (21). FISSH can now offer an invaluable contribution to the differential diagnosis of small round cell tumors. The analysis of single copy DNA sequences in interphase nuclei has already proven to be valuable both for the detection of numerical chromosomal alterations occurring in neoplasia (23–26) and for the detection of specific chromosomal translocations. As far as the molecular study of chromosomal translocations is concerned, FISSH in interphase nuclei has been successfully used for the detection of the

Address correspondence to Dr. Glen A. Evans, Molecular Genetics Laboratory, The Salk Institute for Biological Studies, P. O. Box 85800, San Diego, CA 92138.

Received for publication 5 March 1992 and in revised form 3 June 1992.

J. Clin. Invest.

0021-9738/92/11/1911/08 $2.00
Volume 90, November 1992, 1911–1918

1. *Abbreviations used in this paper:* ES, Ewing's sarcoma; FISSH, fluorescent in situ suppression hybridization; IMDM, Iscove's modified Dulbecco's medium; PN, peripheral neuroepithelioma; PNET, primitive neuroectodermal tumors.

reciprocal translocation (9;22)(q34;q11) associated with chronic myeloid leukaemia, where it was possible to detect the translocation event, even in three cases which had been shown to be cytogenetically Ph-1 negative (27).

In this study, we detected the t(11;22)(q24;q12) in a straightforward manner by means of FISSH on interphase nuclei using the previously described cosmid clones 23.2 and 5.8 mapping, respectively, centromeric and telomeric with respect to the t(11;22) breakpoint on 11q24 and separated by < 1.5 Mb on 11q24 (22). We analyzed four cell lines showing the t(11;22)(q24;q12) and representing different stages of differentiation of PNETs, and 11 patients with small round cell tumors. In three of the patients examined, in which classic cytogenetic analysis had already shown the presence of the consistent t(11;22)(q24;q12), we were able to detect the translocation both in metaphase and interphase preparations. Although cytogenetic analysis provides a complete description of the chromosomal abnormalities present in a given tumor, interphase cytogenetics is an easy and reliable substitute for classical cytogenetic studies for the detection of the t(11;22)(q24;q12).

Methods

DNA probes

Unique copy DNA probes for in situ hybridization were cosmid clones 23.2 (D11S374) and 5.8 (D11S372), located immediately centromeric and telomeric to the t(11;22)(q24;q12) breakpoint on chromosome 11q24. These probes are separated by ~ 1.5 Mb of intervening DNA and were previously shown to detect the t(11;22) in interphase nuclei (22).

Cell lines

Five tumor cell lines were studied in the present work. The characteristics of each cell line are listed in Table I. All cell lines were maintained in Iscove's modified Dulbecco's Medium (IMDM; Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Gibco) and incubated under 5% CO_2 at 37°C.

Short-term cultures from patients' samples

Short-term cultures were established and maintained according to the method of Biegel et al. (8) for cases 1–3 or Fletcher et al. (9). Fresh tumors from cases 4–11 (Table II) were minced in small pieces and incubated 1 h at 37°C in 2 mg/ml collagenase type I (Sigma Chemical Co., St. Louis, MO) in PBS. Every 10 min the solution was vortexed briefly to facilitate the detachment of tumor cells from the stroma. The supernatant was then collected and centrifuged at 1,000 rpm for 10 min and the cell pellet washed twice in IMDM medium containing 15% FCS. Cells were seeded in 25-cm^2 flasks (Corning Glass Inc., Corning, NY) previously coated with fibronectin (Collaborative Research, Waltham, MA), with IMDM supplemented with 15% FCS (Gibco), 1% bovine pituitary extract (Collaborative Research), and 0.5% mito + serum extender (Collaborative Research). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. In most of the cases, cells were harvested by trypsinization within 4 d after the establishment of the culture, washed twice in cold PBS, treated in a hypotonic solution of 0.075 M KCl for 30 min, and fixed with at least three changes of methanol/acetic acid (3:1). In case No. 8 and No. 10 cells were harvested 2 wk after establishment of the culture.

Direct harvesting for interphase nuclei preparations

In cases No. 6 and No. 9, tumor cells obtained from the disaggregation of the biopsy specimen were washed twice in cold PBS, treated in a hypotonic solution of 0.075 M KCl for 30 min, and fixed with at least three changes of methanol/acetic acid (3:1).

Preparation of metaphase chromosomes

Metaphase spreads were prepared following standard techniques (8, 29) with minor modifications.

Fluorescent in situ suppression hybridization

Biotin-labeling. 25–50 ng of DNA were treated for 1 h at 14°C with a dilution 1:1,000 of 1 mg/ml DNAse I. Labeling was then performed using a random primer extension reaction (30), where dTTP was replaced with bio-11-dUTP (Enzo Biochem Inc., New York) at 20 μm final concentration and dCTP was replaced with bio-11-dCTP (Enzo) at 20 μm final concentration, to increase biotin incorporation. The random primer reaction was carried out using a labeling system purchased from Bethesda Research Laboratories (Gaithersburg, MD) according to the manufacturer's instructions. After labeling, the cosmid probes were purified from unincorporated nucleotides by passing the reaction mixture through Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ) in 1-ml spin columns.

Fluorescent in situ suppression hybridization. Before hybridization the metaphase chromosomes were treated as described (22) with some modifications. Slides were treated with RNase (100 μg/ml in 0.3 M NaCl/30 mM sodium citrate – 2× SSC) for 1 h at 37°C. followed by dehydration in a series of 70, 85, and 100% ethanol baths. Proteinase K digestion was then carried out at a concentration of 0.5 μg/ml in 20 mM Tris/2 mM $CaCl_2$ at 37°C for 7 min. Slides were fixed in 4% paraformaldehyde in PBS plus 50 mM $MgCl_2$ at room temperature for 10 min. To obtain chromosome denaturation, slides were immersed in

Table I. Cell Lines

Cell line	Age (yr) /Sex	Tumor site	Diagnosis	Culture (year of establishment)	Karyotype	Reference
6647	14/F	Tibia, prox	Ewing's sarcoma	1974	47,+20,del(X)(q23),i(1q),t(11;22)(q24;q12),22q+	16
TC71	22/M	Humerus	Ewing's sarcoma	1981	76,80,-Y,-8,t(1;7)(q25;p11),del(2)(q36),t(2;14)(q12;q32),3q+,?5,del(6)(q26),del(7)(q31),t(7;11)(q21;q23),t(8;14)(q11;p11),t(11;22)(q24;q12)	16
LAP-35	12/F	Tibia, prox	PNET of bone	1987	48,XX,del(1)(p34),+8,t(11;22)(q24;q12),inv(14)(q11.2:q32),-16,+der(16),t(1;16)(q11;q11.1),+20	29
TC32	17F	Ilium	PN	1979	48,+5,+10,i(1q),t(11;22)(q24;q12)	16
U2OS	15/F	Tibia	Osteosarcoma	1964	Complex abnormalities of every chromosome pair; absence of t(11;22)(q24;q12)	American Type Culture Collection

Table II. Patients

Patient/age (yr)/sex	Tumor site	Diagnosis*	Culture‡	t(11; 22) Cytogenetic analysis	t(11; 22) Interphase cytogenetics (FISSH)
					%
1. 17§/M	Pelvis	ES	1 d	53–60,XY,[+X],+1,+2,[+3],+7,[+7p+], +8,+9,+12,+14,−15,+20,[+20,+20],t(11; 22)(q24;q12),[+t(11; 22)(q24; q12)], + random markers	+(54)
2. 15§/M	Pelvis	ES	4 d	49,XY,+5,+8,+14, inv ins(7) (q32p21 or p15p13), t(11;22)(q24;q12)	+(44)
3. 17§/M	Pelvis	ES	2 d	47,XY,+8,t(11;22)(q24;q12)	+(50)
4. 15‖/M	Lung	ES(met)¶	1 wk	ND	+(16)
5. 9‖/F	Pelvis	ES	1 wk	ND	+(20)
6. 8‖/M	Pelvis	ES	dir	ND	+(54)
7. 18‖/M	Femur, distal	ES	1 wk	ND	+(17)
8. 13‖/F	Femur, proximal	ES	2 wk	ND	+(13)
9. 12‖/M	Femur, distal	SCO	dir	ND	—
10. 10‖/F	Femur, proximal	SCO	2 wk	ND	—
11. 41‖/F	Tibia	CO	1 wk	ND	—

* As determined by light microscopy and immunohistochemistry. SCO; small cell osteosarcoma; CO, chronic osteomyelitis. ‡ Direct harvest (dir) or short-term culture of tissue specimens (weeks). § Children's Hospital, Philadelphia, PA. ‖ Instituti Ortopedici Rizzoli, Bologna, Italy. ¶ Metastasis. [ ] Abnormalities seen only in some cells. Numbers in parentheses are the percentage of nuclei showing the translocation.

70% formamide/2× SSC, pH 7 at 75°C for 2 min, then dehydrated in an ice-cold 70, 85, and 100% ethanol series. Hybridizations were carried out as follows: 25–50 ng of biotinylated cosmid DNA was precipitated with 2 μg of human placental DNA, 1–2 μg of human Alu repeat DNA probe, pBLUR 8 and 7 μg of salmon sperm DNA (22). All competitor DNAs and salmon sperm DNA were ribonuclease treated, extracted with phenol and chloroform, and sonicated to a final size range of 200–400 bp. The biotinylated cosmid probes, coprecipitated with the competitor DNAs, were resuspended in 10 μl of hybridization buffer (50% formamide/2× SSC, pH 7, final concentration, 10% dextran sulfate) and then denatured at 75°C for 5 min. Preannealing of repetitive DNA sequences was carried out for 30 min at 42°C before application of the hybridization mixture to the denatured slides. Hybridization was carried out at 37°C for 16–24 h in a humidified chamber. Posthybridization washes of the slides were carried out as described (22). The slides were then treated with alternating layers of fluoresceinated avidin and biotinylated goat antiavidin (Vector Laboratories, Burlingame, CA), both at 5 μg/ml, as described (22), until two layers of avidin were applied. The avidin and goat antiavidin treatments were separated by three washes of 3 min each in 4× SSC, 4× SSC/0.1% Triton X and PN buffer (0.1 M phosphate buffer pH 8/0.1% Nonidet P-40), respectively. After the final avidin treatment, a fluorescence antifade solution (31) containing 200 μg/ml of propidium iodide to stain double stranded DNA, was applied to the slides under a coverslip.

Microscopy. The slides were evaluated both on a Zeiss Axiophot fluorescence microscope and on a laser scanning confocal microscope (MRC 600; Bio-Rad Laboratories, Richmond, CA) (32, 33) which allows production of higher quality images. Narrow band pass filters were used to obtain separate images of each fluorochrome (550-nm filter for FITC; 610-nm filter for propidium iodide). The two separate images of each object are stored and then overlaid electronically and digital filtering is applied for image optimization. The fluorescence signals are quantitated directly, the signal to noise ratio is enhanced by Kalman optical filtering, using Bio-Rad software, and the images stored in digital form. Color prints from confocal images were produced by a Mavigraph Color video printer (Sony, Tokyo, Japan). Photographs in epifluorescence mode were taken using a Zeiss Axiophot epifluorescence microscope.

Results

In the present study we analyzed by FISSH four PNET cell lines, one osteosarcoma cell line, and 11 patients. 10 patients had small round cell tumors of bone and one had chronic osteomyelitis (Table II). Metaphase spreads from all of the tumor cell lines, and from biopsy specimens of three patients with Ewing's sarcoma were analyzed. For the remaining eight patients comprising five ES (patients 4 to 8), two small cell osteosarcomas (patients 9 and 10), and one chronic osteomielitis (patient 11), only preparations of nuclei were available for analysis. With the exception of cases No. 6 and No. 9, in which direct harvests of nuclei were possible from the biopsy specimens, nuclei were prepared from short term culture. The morphology of these cultures, monitored by phase contrast light microscopy, showed an overgrowth of normal stromal cells as compared to the malignant cells, as the cultures progressed. The heterogeneity of the cellular components in the short-term cultures is in keeping with the finding of variable percentages of nuclei showing the t(11;22)(q24;q12) translocation, ranging from 13 to 54% of nuclei showing four hybridization signals (Table II).

All experiments were observed with an epifluorescence microscope (Axiophot; Zeiss) and with a confocal laser scanning microscope (MRC-600; Bio-Rad). The confocal laser microscope eliminates out-of-focus blur, improves the contrast and sharpness of the image, and allows storage and analysis of all data. Although the use of confocal microscopy was shown to drastically improve the quality of the images, it was not necessary for the diagnostic assessment, which can be easily carried out using epifluorescence microscopy (Fig. 1). After hybridiza-

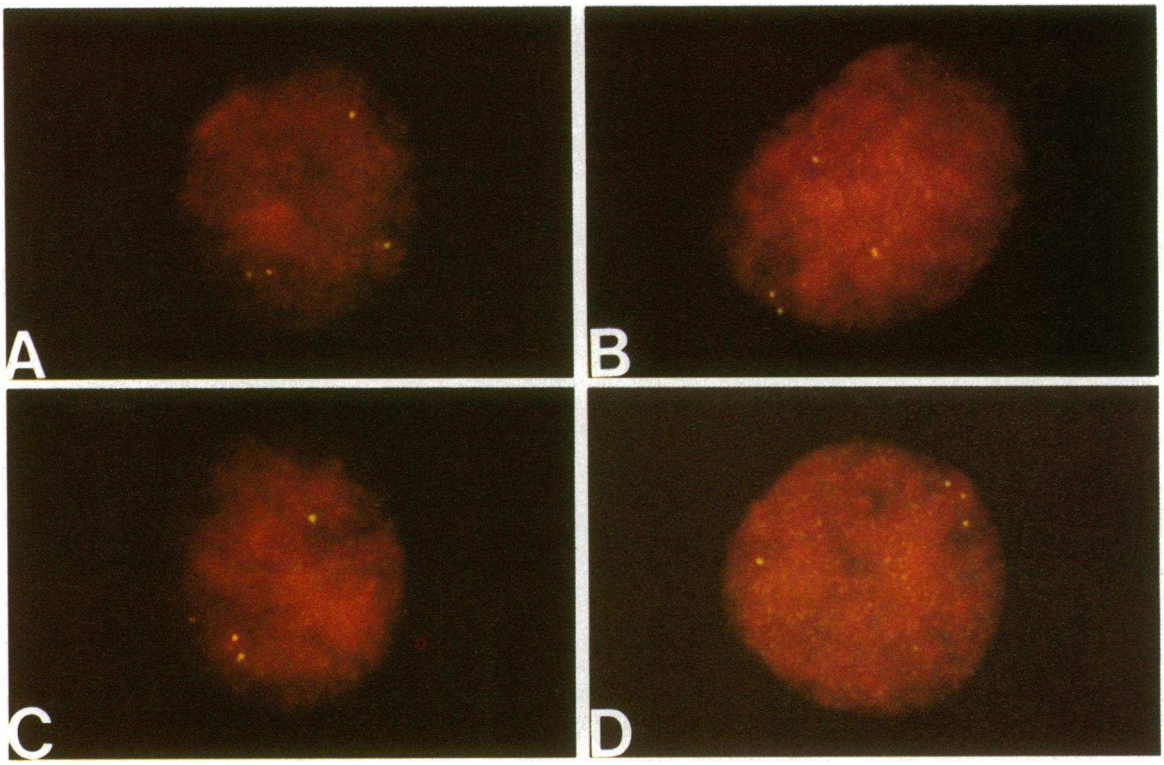

Figure 1. Fluorescent in situ suppression hybridization with cosmid clones 23.2 and 5.8 (22) to interphase nuclei from ES patient 2 (*A*), ES patient 4 (*B*), ES patient 5 (*C*), ES patient 7 (*D*). The photographs were taken using a Zeiss Axiophot epifluorescence microscope (100×).

tion with cosmid clones 23.2 and 5.8 in pairwise combination, both in the cell lines and in the patients' samples, a varying number of nuclei on multiple slides presented four hybridization signals. This is due to many different factors. The nuclei are in different phases of the cell cycle and not all are G_1 arrested, the presence of background due to fluorochromes creates spurious "noise spots" on some areas of the slide, and the efficiency of hybridization varies from slide to slide and within the same slide, from cell to cell. Therefore, only the nuclei clearly presenting four hybridization signals were considered in the present study for the diagnostic assessment of the translocation. Since the study on patients 1, 2, and 3 (Table II) was carried out blind, after the karyotype data were disclosed it was evident that a small percentage of the metaphases of patient 1 presented two t(11;22). This was in keeping with the presence of a small number of nuclei showing six fluorescent signals, but nevertheless it did not change the overall diagnostic conclusion.

FISSH analysis on small round cell tumor cell lines. FISSH analysis with cosmid clones 23.2 and 5.8 was performed on four cell lines derived from ES and PNETs in which the t(11;22)(q24;q12) had been previously described by classic cytogenetic analysis and on one osteosarcoma cell line without the translocation (Table I). Metaphase preparations of the five cell lines were hybridized to cosmid clones 23.2 and 5.8 and hybridization signals were detected in both sister chromatids of normal chromosome 11 and derivative chromosomes 11 and 22, in 90–96% of the metaphases examined, according to the different slides (Fig. 2, *A* and *C*). Cosmid clone 23.2, which had previously been described to map at FLpter 0.98 on normal chromosome 11 (fractional chromosomal length from the end of the short arm; ref. 20, 22), was present on the derivative chromosome 11 at FLpter 0.88, because of the elongation of the chromosome due to the translocated fragment of chromosome 22 (22). Cosmid clone 5.8, which has been mapped at FLpter of 0.98 (22), was found translocated to the derivative chromosome 22. Cell lines without the translocation displayed two hybridization signals on both sister chromatids of both chromosome 11s at FLpter of 0.88 as previously described (22).

In the same preparations, hybridization signals were also observed on interphase nuclei. Up to 58% of the nuclei presenting four hybridization signals showed the translocation by means of a specific hybridization pattern where the derivative chromosomes 11 and 22 were visualized as widely separated single hybridization signals (Fig. 2, *B* and *D*). The remaining 42% of nuclei displayed a hybridization pattern typical of cells without the translocation, i.e., both normal chromosomes 11 were visualized as a pair of closely spaced hybridization signals.

The interphase nuclei of the osteosarcoma cell line without the 11;22 translocation showed two pairs of closely spaced hybridization signals in 99.5% of the cells. In the remaining percentage of the nuclei showing four hybridization signals we

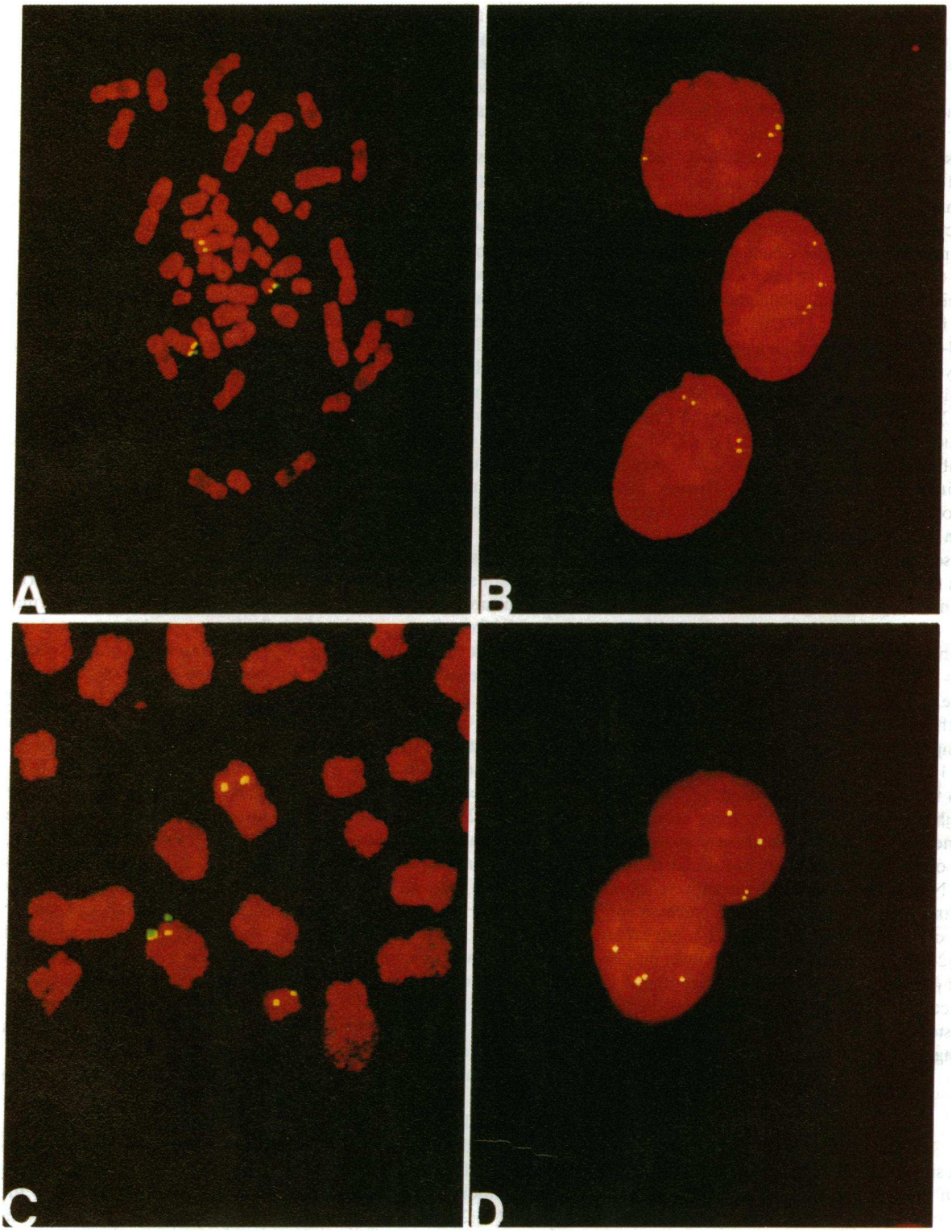

Figure 2. FISSH with pairwise combination of cosmid clones 23.2 and 5.8 (22) to metaphase chromosomes and interphase nuclei from the PNET of bone-derived LAP-35 cell line (28) and from the ES-derived TC71 cell line. Cosmid DNA was labeled with biotin as described in Methods and detected using FITC-avidin. Chromosomes and nuclei were visualized by counterstaining with propidium iodide. Data were collected and images reconstructed using a confocal microscope (MRC-600; Bio-Rad). Color prints were produced by a Mavigraph color video printer (Sony). (*A*) Simultaneous hybridization of cosmid clones 23.2 and 5.8 to metaphase chromosomes from the PNETB cell line LAP-35. The derivative chromosomes 22 (*middle*) and 11 (*upper*) show two hybridization spots, while the normal chromosome 11 (*lower*) shows four spots of hybridization. (*B*) FISSH of clones 23.2 and 5.8 to three interphase nuclei from the PNETB cell line LAP-35. The upper and middle nuclei display the translocation by separation of the two hybridization signals while the lower nucleus does not carry the translocation. (*C*) Simultaneous hybridization of cosmid clones 23.2 and 5.8 to metaphase chromosomes from the ES cell line TC71. The derivative chromosomes 22 (*lower*) and 11 (*upper*) show two hybridization spots, while the normal chromosome 11 (*middle*) shows four spots of hybridization. (*D*) FISSH of clones 23.2 and 5.8 to two interphase nuclei from ES cell line TC71. Both nuclei show the translocation.

observed separation of two of the signals, as seen in the cells bearing the translocation, clearly due to spacial distortion of the nucleus.

FISSH analysis on patients' tumor samples. In three patients affected by Ewing's sarcoma (Table II, Nos. 1, 2, and 3), in which classic cytogenetic analysis had shown the t(11;22)(q24;q12), FISSH with cosmid clones 23.2 and 5.8 in pairwise combination was performed on metaphase preparations. As expected, the translocation was detectable in a straightforward manner in all three cases of Ewing's sarcoma (Fig. 3 *D*). The t(11;22)(q24;q12) was also detectable in the interphase nuclei displaying four hybridization signals, in a percentage ranging from 44 to 54% (Fig. 2, *A–C*). In five patients (Table II, Nos. 4–8) diagnosed as Ewing's sarcoma by means of light microscopy and immunohistochemistry, in which classic cytogenetic analysis had not been possible, we were able to detect the t(11;22)(q24;q12) only by means of FISSH interphase cytogenetics with cosmid clones 23.2 and 5.8 hybridized in pairwise combinations (Fig. 3, *A–C*). With the exception of patient 6 and 9 in which cells were processed immediately after disaggregation of the biopsy specimen, nuclei were prepared from short-term cultures. Interestingly, we found the percentage of cells presenting the translocation inversely affected by culture time. In fact, patients 1, 2, 3, and 6 showed the highest percentage of nuclei presenting the hallmark translocation while patient 8 showed the lowest percentage (Table II). As a matter of fact, the monitoring by phase contrast microscopy of cell morphology in the short-term cultures showed that stromal fibroblasts were gradually overgrowing the malignant cells. This explains the finding of different percentages of normal nuclei presenting four hybridization signals, and obviously not bearing the t(11;22)(q24;q12), present together with the malignant cells on the same slide preparation.

Even though the t(11;22)(q24;q12) has been previously described in one case of small cell osteosarcoma (34), in the two small cell osteosarcoma patients examined in this study (Table II, case Nos. 9 and 10), all the nuclei were found to be normal when analyzed by FISSH with the pairwise combination of cosmid clones 23.2 and 5.8 (Fig. 3 *E*). No evidence of the t(11;22)(q24;q12) was detected in the nuclei preparations from a patient (Table II, No. 1) whose differential diagnosis, based on clinical and radiological examination, included ES and chronic osteomyelitis. After a biopsy was performed, the histological diagnosis of chronic osteomyelitis was established.

Discussion

Fluorescent in situ suppression hybridization in interphase nuclei has proven to be valuable for the study of both numerical and structural chromosomal aberrations in human malignancies (23–27). In this study, we have demonstrated that interphase cytogenetics with FISSH is a useful tool for the detection of the t(11;22)(q24;q12) in small round cell tumors. The previous identification of two molecular probes bracketing the translocation (22) allowed us to visualize it in metaphase chromosomes and interphase nuclei from four cell lines, including two ES (6647, TC71), one PNET of bone (LAP-35), and one PN (TC32) which were previously shown to have the classic t(11;22)(q24;q12) (Table I). An osteosarcoma cell line (U2OS) was included in the study as negative control and was shown not to carry the t(11;22)(q24;q12) by means of cytogenetic analysis (Table I). Analysis of nuclei present in the same preparations clearly showed the translocation by means of a specific hybridization pattern where the normal chromosome 11 was visualized as a pair of closely spaced hybridization signals, while both derivative chromosomes 11 and 22 were visualized as widely separated single hybridization signals.

Interestingly, in the cell lines and short term cultures, the percentage of cells demonstrating the translocation was variable, which is probably due to the presence of more than one cell population. Ideally, interphase cytogenetics should be performed on nuclei in the G_0–G_1 phases of the cell cycle, but this synchronization is not achievable in cultured tumor cells or cell lines where cells are in different phases of the cell cycle and not all are G_1 arrested. Background spots due to fluorochromes and dis-homogeneity in hybridization are also factors that hinder proper interpretation of the results.

Even though most of our present study was carried out using confocal microscopy, we have shown that even normal epifluorescence microscopy can be used for a rapid and easy assessment of the t(11;22)(q24;q12). By analyzing the cell lines by FISSH we established that interphase cytogenetics is a rapid alternative to chromosomal analysis. In view of these results, we further studied nuclei preparations directly obtained from small round cell tumor patient biopsies and short term cultures. We were able to detect the t(11;22)(q24;q12) in nuclei from three cases in which the karyotype demonstrated the translocation and in five cases of Ewing's sarcoma in which cytogenetic analysis had not been possible (Table II, Nos. 4–8). Two cases of small cell osteosarcoma (Table II, patient 9 and 10) were also included in this study and they were both normal with respect to the t(11;22)(q24;q12). One patient (Table II, No. 11) in which the differential diagnosis had been particularly intriguing, did not show any detectable evidence of t(11;22)(q24;q12) by FISSH interphase cytogenetics. On the grounds of histological examination of the biopsy specimen, this patient was ultimately diagnosed with chronic osteomyelitis of the tibia. Interphase cytogenetic analysis carried out by FISSH with cosmid clones 23.2 and 5.8 was also of particular value in the final diagnosis of patient 9 (Table II), a 12-yr-old boy with a neoplasm of the distal femur, whose differential diagnosis included Ewing's sarcoma and small cell osteosarcoma. Light microscopic examination and immunohistochemistry, together with the negative FISSH interphase result, was diagnostic for a small cell osteosarcoma.

An interesting correlation was found between time (days) of in vitro culture and ratio of cells displaying the chromosomal abnormality. In fact, nuclei obtained from cells maintained in culture for longer periods displayed an increased number of normal diploid cells. Therefore, direct harvesting from tumor biopsies seems to be preferable for detecting the t(11;22)(q24;q12) in interphase nuclei by FISSH. This eliminates the need of the cumbersome in vitro culture techniques required by the classic cytogenetic analysis, and would theoretically be possible from very small tumor specimens such as needle biopsies. If FISSH reveals the characteristic translocation, the small round cell tumor is highly likely to be an ES. If the breakpoint is not detectable by FISSH this may simply be for technical reasons, which can be overcome by repeating the analysis of that sample, or it may be due to the absence of the t(11;22). However, it is known that a small percentage of ES do not bear the t(11;22)(q24;q12), as far as classic cytogenetic analysis shows (15). In these cases a definite differential diag-

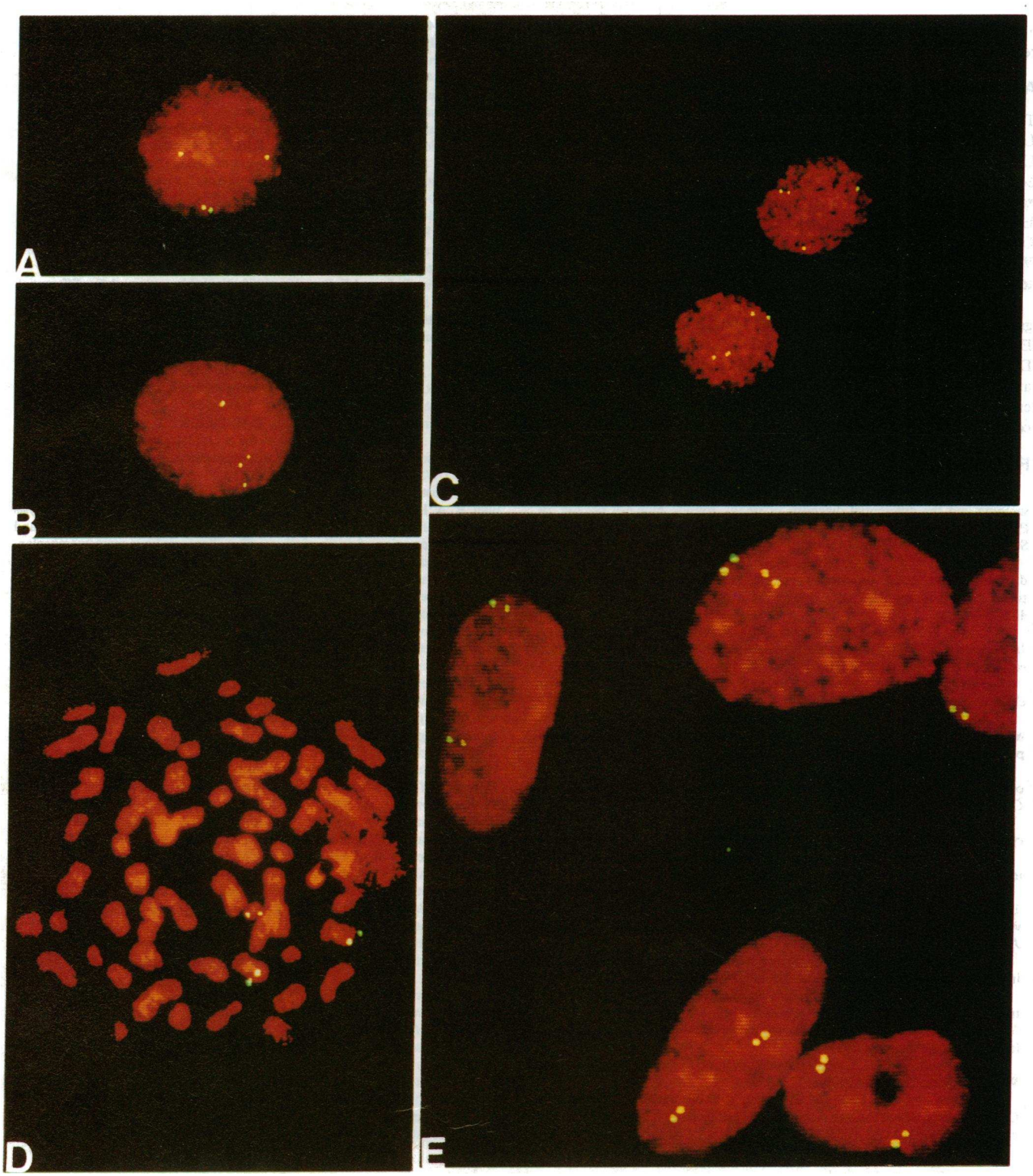

Figure 3. FISSH with cosmid clones 23.2 and 5.8 (22) to metaphase chromosomes and interphase nuclei from ES and small cell osteosarcoma patients. (*A*) Interphase nucleus from ES patient 5. (*B*) Interphase nucleus from ES patient 6. (*C*) Two interphase nuclei from ES patient 7. Only the upper nucleus carries the translocation, the other being normal. (*D*) Metaphase spread from ES patient 1 showing hybridization spots on both 11 (*lower*) and 22 (*upper*) derivative chromosomes and on normal chromosome 11 (*right*). (*E*) Interphase nuclei from a patient with small cell osteosarcoma (Table II, no. 9), all the nuclei are normal with respect to the t(11;22)(q24;q12).

nosis will be reached only by the simultaneous analysis of all data provided by clinical features, immunohistochemistry, and electron microscopy.

We predict that interphase cytogenetics will be carried out in the future on needle biopsy specimens and hopefully on paraffin-embedded tissue samples, and will become a funda-

mental tool for the differential diagnosis among small round cell tumors.

Acknowledgments

The authors want to thank Dr. Timothy J. Triche (Children's Hospital, Los Angeles, CA) and Dr. Gian Paolo Bagnara (Institute of Histology, University of Bologna, Italy) for providing cell lines, Dr. Massimo Serra, Dr. Nicola Baldini and Dr. Piero Picci (Istituti Ortopedici Rizzoli, I. O. R., Bologna, Italy) for providing tumor samples, Dr. James Eubanks for assistance in confocal microscopy, Dr. Mary Saleh, Dr. Gary Hermanson, Dr. David Mc Elligott, Dr. Malek Djabali, Caryn Wagner-Mc Pherson for helpful discussion, and Annette Parmiter for technical assistance.

This work was supported by National Institutes of Health (NIH) grants CA 47983 (J. A. Biegel and B. S. Emanuel) and CA 39926 (B. S. Emanuel), GM33868 and HG00202 (G. A. Evans) from the NIH, the Department of Energy (G. A. Evans), and funds from the G. Harold and Leila Y. Mathers Charitable Foundation. M. Giovannini is a recipient of an Italian Research Council (Consiglio Nazionale delle Ricerche) fellowship. L. Selleri is a recipient of an NIH Fogarty Award.

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