



# Fluorescence In Situ Hybridization-Based Approaches for Detection of 12p Overrepresentation, in Particular i(12p), in Cell Lines of Human Testicular Germ Cell Tumors of Adults

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**ABSTRACT:** Overrepresentation of the short arm of chromosome 12 is frequently detected in human testicular germ cell tumors of adolescents and adults (TGCT). This overrepresentation mostly results from the formation of an isochromosome: i(12p). Whether the overrepresentation consistently involves the complete 12p arm including the centromere is still unclear. We studied five TGCT-derived cell lines (NT2, 2102Ep, H12.1, NCCIT, and S2), combining conventional chromosome banding, fluorescence in situ hybridization (FISH), and comparative genomic hybridization (CGH) to investigate the suitability of each of these techniques to detect aberrations involving chromosome 12. Karyotyping showed one or more i(12p)s in NT2, 2102Ep, H12.1, and S2. However, FISH with a centromere-specific probe ( $\alpha$ 12H8), a 12p "paint" and a 12p11.2-p12.1 region-specific probe yeast artificial chromosome (YAC) #5 and CGH could not confirm the presence of an i(12p) in S2. Additional randomly distributed 12p sequences were detected by FISH in H12.1, NCCIT, and S2. In most of these cases, (a part of) the centromere was included. All overrepresented 12p regions, except for those in S2, showed hybridization with YAC#5. CGH showed increased copy numbers of the complete 12p arm in the cell lines with one or more i(12p)s but no overrepresentation was noted in the cell lines without i(12p). In metaphase spreads, the centromeric block of the i(12p)s differed in size as compared with those of normal chromosomes 12. This was rarely noted in interphase nuclei. A decrease in size of the centromeric block in 2102Ep and H12.1 caused a weak FISH signal, which was difficult to detect, especially in interphase nuclei. The ratio between  $\alpha$ 12H8- and YAC#5-derived signals reflected the presence or absence of one or more i(12p)s. Our results indicate that double FISH with a centromere- and a 12p-specific probe can be used to detect 12p overrepresentation [including i(12p)] in TGCT both in metaphase spreads and interphase nuclei. CGH confirmed the relative overrepresentation of 12p sequences as detected by FISH and showed that in these cell lines the complete 12p was involved.

## INTRODUCTION

Human testicular germ cell tumors of adolescents and adults (TGCT) can clinically and histologically be grouped

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into two entities; the seminomas (SE) and nonseminomatous TGCT (NS) [1, 2]. All TGCT originate from a common precursor, carcinoma in situ (CIS) [3]. Despite a hypertriploid DNA content for CIS and SE and a hypotriploid DNA content for NS [4-6], they show a striking similarity in chromosome constitution [7], supporting a common pathogenetic origin. The only recurrent chromosome abnormality detected both in SE and NS by conventional karyotyping is an isochromosome of the short arm of chromosome 12 [i(12p)], present in ~70% of all TGCT [8]. The presence of an i(12p) is sufficiently specific to classify a cancer of the male gonad as a TGCT [9, 10]. In addition, the number of copies of i(12p)s has been suggested to be a prognostic

parameter [11, 12]. Cytogenetically, TGCT without i(12p) show significantly more breakpoints in the 12p13 band as compared with those with i(12p) [8, 13].

Recently, fluorescence *in situ* hybridization (FISH), which can be applied on metaphase spreads as well as on interphase nuclei ("interphase cytogenetics"), was introduced [14–17]. This technique confirmed the genuine nature of the i(12p) in TGCT [18, 19] and showed that all i(12p)-negative TGCT tested so far contained additional 12p sequences [13, 20], implying that relative overrepresentation of 12p sequences is crucial for the development of a clinically manifest TGCT. FISH on interphase nuclei with a centromere-specific probe for chromosome 12 has been used to identify TGCT [11, 21], based on a consistent size difference between the hybridizing centromeric region of the isochromosome and the normal chromosome 12 homologues. The reliability of this method depends on the consistent involvement of centromeric sequences in the formation of i(12p). TGCT without size differences of the centromeric regions of the chromosomes 12 and their derivatives, including the "i(12p)-negative" TGCT, will not be recognized by this approach.

We used the combination of chromosome banding and FISH on metaphase spreads to study overrepresentation of centromeric and 12p sequences in five TGCT cell lines. The results were compared with the findings on interphase nuclei. In addition, we used the comparative genomic hybridization (CGH) technique [22, 23] to study the presence of 12p overrepresentation in these cell lines.

## MATERIALS AND METHODS

### Cell Lines

We analyzed five established TGCT-derived cell lines, three derived from NS (i.e., NT2 [24], 2102Ep [25], and H12.1, a gift from H.-J. Schmoll, Hannover, Germany) and two cell lines reported to show SE-like characteristics (i.e., NCCIT [26] a gift from I. Damjanov, TX, U.S.A.) and S2 (a gift from A. von Keitz, Marburg, Germany). The cell lines were cultured by conventional methods (37°C, 5% CO<sub>2</sub>) in culture flasks (Costar, Cambridge, England) and passaged every 2–4 days by trypsinization, depending of the growth rate of each individual cell line.

### Slide Preparation for Conventional Chromosome Banding and FISH

Cell suspensions for generation of metaphase spreads of the five cell lines were prepared according to standard procedures. The mitotic cells were harvested after 2–4 h of Colcemid (Life Technologies, NY, U.S.A.) treatment, swollen in hypotonic KCl solution, and fixed with methanol:acetic acid fixative.

For conventional karyotyping, air-dried preparations were banded with pancreatin (Sigma, St. Louis, MO, U.S.A.) as reported previously [27]. The chromosome constitution is described according to the International System for Human Cytogenetic Nomenclature [28], except that it is calculated on the basis of a triploid instead of a diploid DNA content because of the consistent peritriploid DNA content of TGCT [4, 7].

The slides used for the combination of GTG-banding and FISH were prepared as reported previously [29], with some modifications. Air-dried slides were incubated overnight at 60°C. During the first minutes, the temperature was continuously raised from room temperature to the final temperature. Subsequently, the slides were washed in 2 × SSC for 1.5 h. After a single wash in 0.85% NaCl, the slides were digested for 1–5 s at room temperature with 0.25% trypsin (Difco, Brunswick, Amsterdam, The Netherlands) in the same buffer. After two washes in 0.85% NaCl, they were stained for 3 min with Giemsa, according to the manufacturer's recommendations (Brunswick Chemie, Amsterdam, The Netherlands). The slides were washed twice and air dried. Metaphases of interest were photographed with an Axiophot microscope (Zeiss, Weesp, The Netherlands) and then destained in 70% ethanol for 15 min at room temperature and directly used for FISH.

### Generation and Labeling of the Probes for FISH

To obtain a suitable 12p "paint" for FISH, i(12p)s from the cell line NT2 were flow sorted, amplified, and biotin labeled by degenerated oligonucleotide primed (DOP)-polymerase chain reaction (PCR) as previously described [30]. In addition, a yeast artificial chromosome (YAC)#5 (a gift from Dr. R. Gemmill, Denver, CO, U.S.A.), mapped to chromosome region 12p11.2–p12.1 [23] was used. This YAC, ~300 kilobases (kb) long, was purified by pulse-field gel electrophoresis and amplified by DOP-PCR (38 cycles) as described previously [31]. Subsequently, the DNA was labeled with biotin-14-dUTP (GIBCO-BRL, Gaithersburg, MD, U.S.A.) in a second round of amplification (30 cycles) under the same conditions, except that the dTTP concentration was reduced to half. The centromeric region of chromosome 12 was visualized with probe p $\alpha$ 12H8 [32, 33], which was labeled by a standard nick-translation kit (Boehringer, Mannheim, Germany) with biotin-11-dUTP (GIBCO-BRL) for the single and with digoxigenin-11-dUTP (Boehringer) for the double FISH experiments.

### FISH

The labeled probes were dissolved separately in 10  $\mu$ l hybridization mixture (hybmix), 2×SSC containing 50% formamid (Merck, Darmstadt, Germany), 10% dextran sulfate (Pharmacia, Uppsala, Sweden), and 5 mg/ml herring sperm as carrier DNA in 2×SSC (final pH 7.0). The probe concentrations in the hybmix were 2 ng/ $\mu$ l for p $\alpha$ 12H8 and 20 ng/ $\mu$ l for both the 12p paint and YAC#5. FISH was performed as described, previously [14], with some minor modifications. After denaturation (70°C for 5 min in hybmix), the 12p paint and purified YAC#5 were preannealed with a 25-fold excess of COT-1 DNA (Life Technologies). For the double FISH, the heat-denatured (100°C for 3 min) p $\alpha$ 12H8 was added to the hybmix after preannealing of YAC#5. The denatured probe mix was added to denatured slides (4 min in 70% formamid at 72°C, 2×SSC, pH 7.0) and hybridized for 16 h (overnight).

The hybrids were visualized with mouse-anti-digoxigenin, tetramethylrhodamine isothiocyanate (TRITC) conjugated rabbit-anti-mouse and TRITC-conjugated goat-anti-rabbit (all Sigma) or alternating layers of FITC-conjugated

avidin and biotinylated goat-anti-avidin antibodies (Vector Laboratories, Burlingame, CA, U.S.A.). Finally, the slides were mounted in antifade (*p*-phenylenediamine dihydrochloride, 90% glycerol, pH 8.0), supplemented with 4,6-diamino-2-phenylindole (DAPI, Sigma) (final concentration 1 µg/ml) for counterstaining of the chromosomes. Results were studied with a Zeiss Axiophot epifluorescence microscope, equipped with appropriate filters for the visualization of FITC, TRITC, and DAPI fluorescence. Representative photographs were made conventionally or with a Photometrics high-performance CH250/A cooled CCD-camera (Photometrics, Tucson, AZ, U.S.A.). The final figures were generated with a Macintosh Quadra 950 computer using the BDS-image FISH software package (Oncor, Gaithersburg, MD, U.S.A.).

### Screening and Interpretation

The karyotypes were interpreted by an experienced cytogenetic technician unaware of the FISH results. FISH results were scored separately by two individuals. For each experiment, 25 metaphases and 100 interphase nuclei were counted. Signal distributions per sample were sum-

marized as the mean number of spots/metaphase (MNSM) or interphase nucleus (MNSI), and SD was calculated. The differences in sizes of the fluorescent centromeric block of the normal and chromosome 12 derivatives, including i(12p), were also scored. Statistic analysis was performed with the unpaired Student's *t* test.

### CGH

CGH was performed on conventionally prepared slides for karyotyping as described previously [22, 23, 34, 35]. The metaphases as well as control DNA for the hybridization were obtained from a normal male individual. For each hybridization, 400 ng tumor DNA was labeled with digoxigenin and a similar amount of control DNA was labeled with biotin. COT-1 DNA 80 µg (Life Technologies) was added to reduce background signal due to repetitive sequences. After incubation for 2–4 days under a coverslip in a moist chamber, the slides were washed by procedures described for FISH. The hybrids were visualized with FITC-conjugated sheep-antidigoxigenin (Boehringer) (tumor specific signal), and pentamethine cyanine dye isothiocyanate (CY3) conjugated avidin (Jacksons, Immuno Research,

**Table 1** Modal composite karyotypes of the testicular germ cell tumor derived cell lines studied<sup>a</sup>

Cell line	Chromosomal constitution
NT2	56–61,add(X)(q24),der(X)t(X;1)(q13;q11),+der(X)t(X;1)(p11;p22),-Y,del(1)(p36),i(1)(p10),add(2)(q34),-4,-5,-6,add(6)(q25),der(7)t(7;17)(q22;q21),-8,add(9)(q21),-10,add(10)(p11.1),-11,der(11)t(11;15)(q11;q15),+i(12)(p10)x2,-13,add(13)(q21),-14,-15,-17,-18,-19,add(20)(p13),-21,-22,-22,+6-8mar[cp9]
2102Ep	51–56,XX,-Y,dup(1)(q11q21),-2,-3,del(3)(q11),-4,-5,-6,dic(7;9)(q11.2;p13),-8,add(8)(p11),-9,-9,-10,del(11)(q13q21)x2,del(12)(q22),+der(12)t(3;12)(q11;q11),+i(12)(p10),-13,-13,-14,add(14)(p11.1),-15,add(16)(q24),-17,-18,-19,-21,-22,-22,+5-10mar[cp10]
H12.1	Clone A: 52–55,XX,-Y,der(1)t(1;7)(p32;p15),-2,add(2)(p25),-3,add(3)(q29),-4,-5,-6,del(6)(q16),add(7)(q21),-8,-9,-10,add(10)(p15),der(11)add(11)(q13)del(11)(p15),+i(12)(p10)x2,-13,der(13)t(13;13)(p12;q12),-14,add(14)(p11),-15,-15,-16,-17,der(17)t(17;17)(q11;p12),-18,add(18)(p11.1),-19,-19,-20,der(20)t(8;20)(q13;q13),-21,-22,+4-7mar[cp4] Clone B: 57-59,XX,+der(X)t(X;10)(q25;q21),-Y,der(1)t(1;7)(p32;p15),dup(1)(q11q21),-2,add(2)(p25),-4,-5,del(6)(q16),+add(7)(q21),-8,add(8)(p11),-9,-9,-10,-11,-12,i(12)(p10),+i(12)(p10),-13,-13,-14,-14,-15,add(16)(q24),-17,-18,-18,-19,-20,-22,+5-7mar[cp2] Clone C: 53–57,XX,+der(X)t(X;10)(q25;q21),-Y,dup(1)(q11q21),-2,-2,-3,-5,-6,add(8)(p11),-9,-9,-10,-11,del(12)(q22),+i(12)(p10)x2,-13,-13,-14,-15,-18,-19,-21,-22,+2-4mar[cp2]
NCCIT	53–57,add(X)(p22.1),add(X)(p22.1),-Y,add(1)(p21),del(1)(p21),+del(1)(q24),+del(1)(q31),add(3)(q11),-4,del(4)(p15.2p15.3),-6,add(6)(p11.2),del(6)(p23),-7,add(7)(p15),del(7)(q11.2),-8,del(8)(p22),-9,-9,-10,-10,add(11)(p11.2),der(12)t(9;12)(q12;q12),-13,-14,add(14)(p11),-15,der(15)t(15;?)(p11.1;q11),-16,-17,-18,der(19)t(7;19)(q11.2;p13.1),-20,-21,-22,-22,+der(?)t(?)1(?)p13),+der(?)t(?)10(?)q11.2),+4-6mar[cp10]
S2	54–57,X,der(X)t(X;8)(p11;p11),-Y,-1,-1,add(2)(p25)x2,add(2)(p16),-3,-3,add(3)(q11),-4,-4,der(4)t(4;12)(p13;q11),-5,-5,-5,add(6)(q11),del(6)(q2?),-8,del(8)(p21),-9,-10,-10,-10,der(11)t(5;?)(q13;?)(p11),der(12)t(8;12)(q13;q24)x2,i(12)(p10),-13,-14,add(14)(p11),-15,add(15)(p11),der(15)t(5;15)(q13;p12),-16,-17,-18,-18,der(19)t(1;19)(p12;p13.2),-20,add(20)(q13.3),+add(21)(p12),-22,-22,+der(?)t(?)1(?)q21),+der(?)t(?)3(?)p11),+8-12mar[cp7]

<sup>a</sup>Descriptions are based on a triploid DNA content.

West Grove, U.S.A.) (control signal). The results were evaluated with a Zeiss Axiophot epifluorescence microscope, equipped with a Photometrics high-performance CH250/A cooled charge-coupled device (CCD)-camera (Photometrics) connected to a Macintosh Quadra 950 computer using the comparative genomic hybridization applications provided in the BDS-image FISH software package (Oncor). From each cell line, at least 10 metaphase spreads with similar imbalances on both chromatids of both chromosome 12 homologues were interpreted. The individual chromosomes were identified by computer images obtained from the DAPI-banded metaphase chromosomes. Digital analysis allowed representation of the results as fluorescence intensity profile for each fluorochrome along the chromosome. The average green/red ratio was calculated; peaks in the green profile were interpreted as overrepresentation. Photographs were made directly from the computer screen.

## RESULTS

Conventional chromosome banding was performed on five TGCT-derived cell lines. The results are shown in Table 1 as modal composite karyotypes. Three different clones were detected in H12.1. In the context of this study, we focus only on chromosome 12 and related aberrations. NT2, 2102Ep, H12.1, and NCCIT showed at least two normal chromosomes 12. An i(12p) was identified in NT2 (2), 2102Ep (1), H12.1 (2) and S2 (1). No i(12p) was noted in NCCIT, but this cell line showed a der(12)t(9;12)(q12;q12). S2 contains two der(12)t(8;12)(q13;q24) and one der(4)t(4;12)(p13;q11), and 2102Ep showed a der(12)t(3;12)(q11;q11) and a del(12)(q22). The latter anomaly was also present in H12.1. Conventional chromosome analysis indicated that three copies of the short arm of chromosome 12 were present in NCCIT, 4 were present in S2, 6 and 7 were present in H12.1, 6 were present in 2102Ep, and 7 were present in NT2. The expected copy numbers of centromeric regions are therefore, 3, 3, 5, 5, and 5, respectively.

For the simultaneous detection of centromeric and 12p sequences, double FISH with  $\alpha$ 12H8 and YAC#5 was performed. In this approach,  $\alpha$ 12H8 was labeled with digoxigenin (detected with TRITC) and the YAC with biotin (detected with FITC). In addition,  $\alpha$ 12H8 labeled with biotin was used in single FISH. The mean numbers of signals per metaphase spread and interphase nucleus (and corresponding SD) of single and double FISH are shown in Table 2. The FISH patterns are shown in Fig. 1 and representative examples are shown in Fig. 2. The results obtained from NT2 and NCCIT confirmed the findings of karyotyping. In contrast, FISH showed that the cytogenetically defined i(12p) in S2 contained no chromosome 12-derived sequences and was therefore incorrectly identified as such (Fig. 1A). In addition, FISH showed that der(4)t(4;12)(p13;q11) must be reclassified as der(12)t(4;12)(q12;p11.2)(Fig. 1B).

Despite lack of hybridization of YAC#5 with one of the chromosome 12 derivatives in S2, the 12p paint recognized a small region just proximal to the centromere (Fig.

**Table 2** Summary of the single and double FISH results using the centromere-specific probe  $\alpha$ 12H8 and YAC#5 for the 12p11.2–p12.1 subband on metaphases and interphase nuclei of five TGCT-derived cell lines

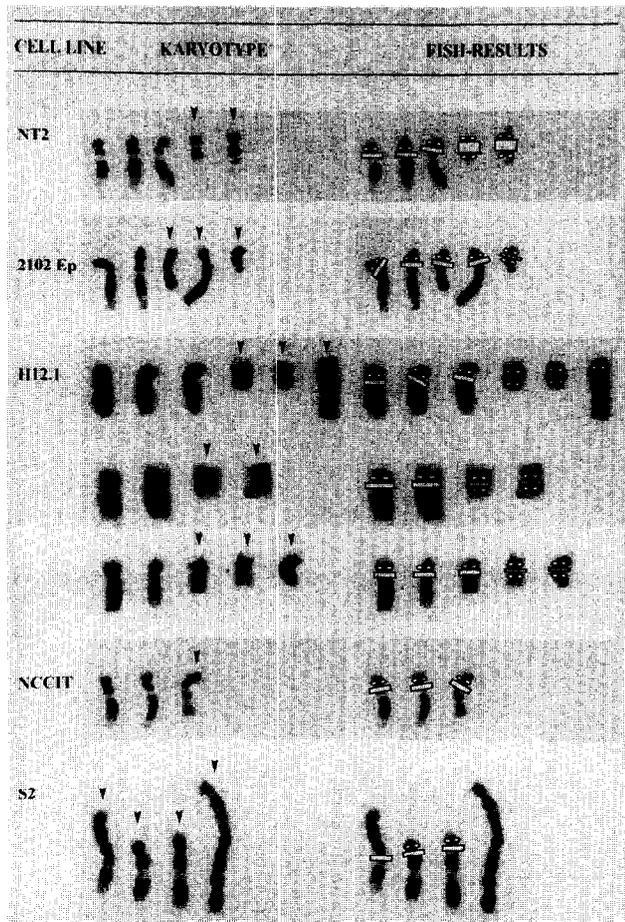
Cell line	Single FISH	Double FISH		Ratio YAC/ $\alpha$ 12H8
	$\alpha$ 12H8	$\alpha$ 12H8	YAC	
NT2				
MNSM	5.0 (0.3)	4.7 (0.6)	6.7 (0.4)	1.4
MNSI	5.0 (1.0)	4.2 (0.7)	6.1 (0.9)	1.5
2102Ep				
MNSM	3.9 (0.7)	4.0 (0.7)	5.8 (0.9)	1.5
MNSI	3.3 (0.5)	2.8 (0.8)	5.3 (1.1)	1.9
H12.1				
MNSM	4.5 (0.6)	4.1 (0.5)	5.2 (0.6)	1.3
MNSI	3.9 (0.8)	3.4 (1.0)	4.3 (1.0)	1.3
NCCIT				
MNSM	2.9 (0.5)	2.8 (0.7)	2.8 (0.8)	1.0
MNSI	3.3 (0.7)	2.7 (0.7)	2.8 (0.8)	1.0
S2				
MNSM	3.0 (0.2)	2.9 (0.3)	2.0 (0.0)	0.7
MNSI	3.2 (0.7)	2.3 (0.7)	1.9 (0.3)	0.8

*Abbreviations:* FISH, fluorescence in situ hybridization; MNSM and MNSI, mean number of spots per metaphase and per interphase nucleus; YAC, yeast artificial chromosome.

The centromeric probe was labeled with biotin for the single and with digoxigenin for the double FISH. The MNSM and MNSI as well as corresponding SD (in brackets) are indicated. The ratio between the numbers of YAC- and  $\alpha$ 12H8-derived signals for each cell line is shown.

1B). In addition, in this cell line cryptic 12p sequences were detected in der(?)t(?)3)(?)p11) and in the cytogenetically identified add(18)(p11.1) in H12.1. Analysis showed that this latter region also contained a small  $\alpha$ 12H8-hybridizing fragment (Fig. 1C), as well as a region recognized by YAC#5 (data not shown). In contrast, no YAC#5 hybridization was detected in the aforementioned 12p-derived region in S2 (data not shown). The 12p sequences other than those present in normal copies of chromosome 12 and i(12p)s were distributed randomly throughout the genome, i.e., associated with (parts of) chromosomes 3, 4, 8, 9, 18, and 19.

No size differences between the fluorescent centromeric regions of the normal chromosomes 12 and its derivatives were detected in NCCIT and S2. In contrast, on metaphase spreads, both i(12p)s in NT2 contained an enlarged centromeric region (Fig. 1D), whereas the i(12p)s in 2102Ep and H12.1 contained a smaller hybridizing region. These size differences could only be identified on <5% of the interphase nuclei whether biotinylated or digoxigenin-labeled  $\alpha$ 12H8 was used, as illustrated by Fig. 1E, which shows nucleus of NT2 hybridized simultaneously with  $\alpha$ 12H8 and YAC#5. The appearance of the different signals indicates the presence of three normal chromosomes 12 and two i(12p)s. The  $\alpha$ 12H8-hybridizing region in add(18)(p11.1) of H12.1 already mentioned and the region present in der(12)t(3;12)(q11;q11) of 2102Ep were reduced in size as compared with their normal homologue (not shown). Despite multiple attempts, the latter centromeric region could be detected by FISH in only 30% of the metaphase



**Figure 1** Fluorescence in situ hybridization (FISH) results in chromosome 12 and derivatives (arrow) with the centromeric probe  $\alpha$ 12H8 (indicated by a block) and yeast artificial chromosome (YAC)#5 (circles).

spreads. Because of the involvement of the centromere of chromosome 12 in this translocation, it must be reclassified as  $\text{der}(12)\text{t}(3;12)(\text{q}11;\text{q}10)$ .

In metaphase spreads of 2102Ep, NCCIT, and S2, similar numbers of centromeric regions were detected with the biotinylated and digoxigenin-labeled  $\alpha$ 12H8 probe (Table 2). Lower copy numbers were observed in NT2 and H12.1 when the latter was compared with the former ( $p < 0.05$  and  $0.02$ , respectively). On interphase nuclei, this decrease in copy numbers was significant in all cell lines ( $p < 0.001$ ). In general, a lower number of centromeric- and 12p-specific signals was observed on interphase nuclei as compared with metaphase spreads. This was mainly true of the centromeric region in the cell lines showing a reduced size of the fluorescent signal of the chromosome 12 derivatives (2102Ep and H12.1).

Single hybridization with  $\alpha$ 12H8 showed chromosome 12-centromeric regions of more than two in all cell lines tested. Distinction between a normal chromosome 12, an  $i(12p)$ , or another chromosome 12 derivative could not be made with this approach on interphase nuclei. The simultaneous application of  $\alpha$ 12H8 and YAC#5 indicated

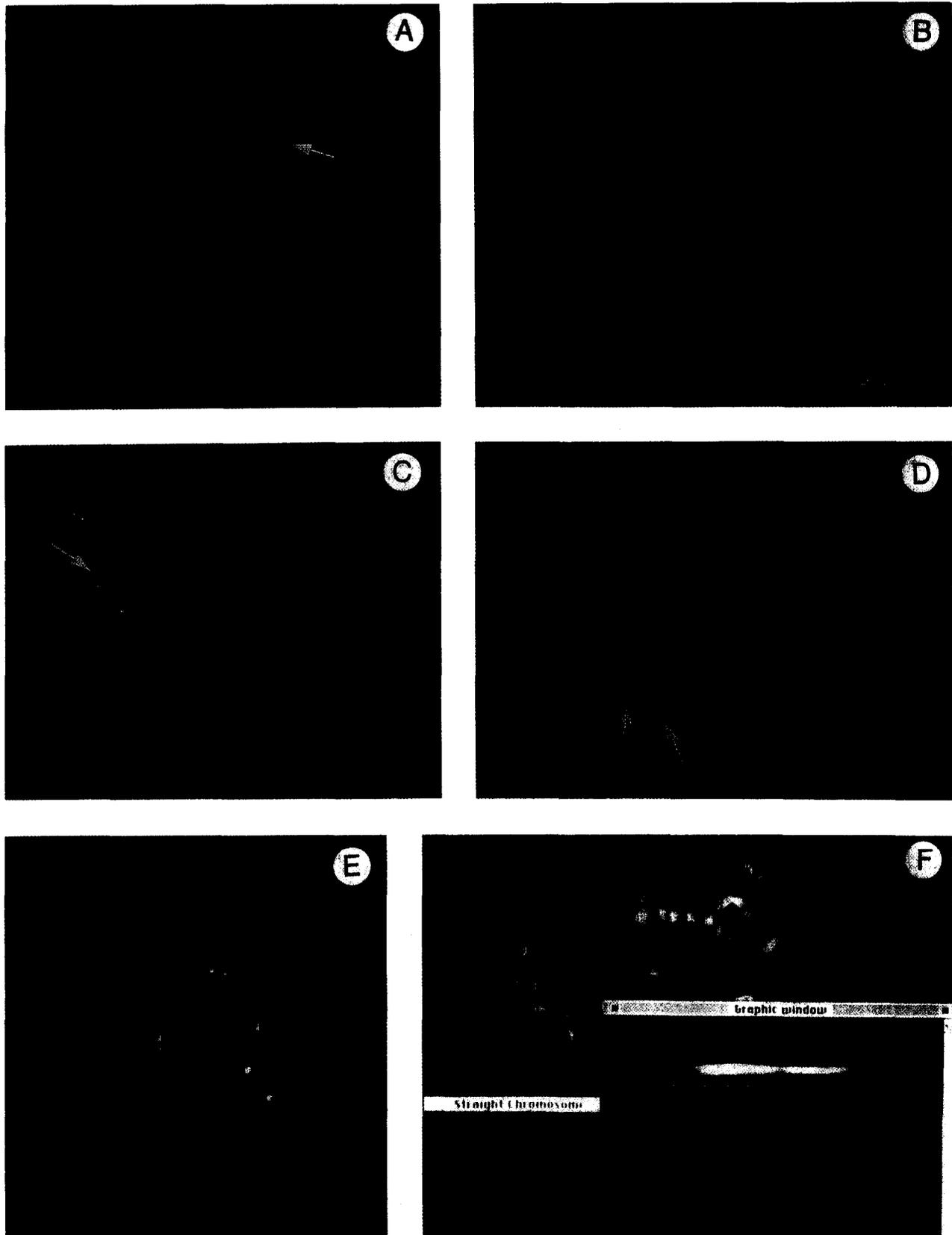
a relative 12p overrepresentation as compared with the centromeric regions in all three  $i(12p)$ -containing cell lines (NT2, 2102Ep, and H12.1), i.e., ratio of YAC#5 to  $\alpha$ 12H8 of  $\geq 1.3$ . The cell lines with no  $i(12p)$  (NCCIT and S2) had a ratio of  $\leq 1.0$ .

To study the value of CGH for the detection of 12p overrepresentation in TGCT in comparison to conventional cytogenetic and FISH analysis, CGH was used on the five cell lines included in this survey. The analysis was based on a triploid DNA content of the tumor cells, without contamination of the sample with host cells. No 12p overrepresentation was detected in the cell lines NCCIT and S2. This finding is in agreement with the combined results obtained by cytogenetic and FISH analyses, showing no more than three copies of the short arm of chromosome 12 in these cell lines. The inability to detect the underrepresentation of 12p sequences in S2, most probably due to the sensitivity of this method, is noteworthy. The other three cell lines (NT2, 2102Ep, H12.1) showed overrepresentation of the complete short arm of chromosome 12 by CGH (shown for NT2 in Fig. 1F), in accordance with the results of cytogenetic analysis combined with FISH, showing six or more copies of 12p per nucleus.

## DISCUSSION

After the first report of the presence of an  $i(12p)$  in TGCT in 1982 [36], multiple studies of this isochromosome were published, dealing with the possible clinical implications [37] as well as different detection methods. The latter include conventional karyotyping, molecular and FISH strategies. Cytogenetically,  $i(12p)$  can be detected in most TGCT [7, 38, 39], supported by molecular data [39, 40]. The FISH approach [11] is based on the use of a centromere specific probe for chromosome 12. This probe has been reported to detect a consistent size difference between the hybridizing region of a normal chromosome 12 and an  $i(12p)$ . Because of a discrepancy in the literature regarding this phenomenon [18–20, 23, 41], as well as the occurrence of TGCT without  $i(12p)$  [13, 20, 42], we studied the possibility of identifying 12p overrepresentation in general, and of  $i(12p)$  in particular, on metaphase spreads and interphase nuclei with a double FISH approach. In addition, CGH was applied to investigate whether the entire 12p arm was overrepresented. Because cell lines, in contrast to tumor samples, enable a detailed description of the chromosome constitution and comparison of the data obtained from metaphase spreads and interphase nuclei, five TGCT-derived cell lines were included in this study; three NS (NT2, 2102Ep, and H12.1) and two cell lines reported to be SE-like (NCCIT and S2). Ours is the first study in which the reproducibility and sensitivity of different methods in detecting chromosome 12 aberrations in TGCTs was tested in detail.

Conventional karyotyping in combination with FISH showed the presence of  $i(12p)$  in all three NS cell lines but not in the two with a SE-like phenotype, which is of interest because  $i(12p)$  is more frequently detected in NS as compared with SE; 83 versus 56% in the largest series of primary TGCT (102) analyzed so far [8]. The aberrant size



**Figure 2** (A) Double fluorescence in situ hybridization (FISH) of a metaphase spread of S2 with the centromere-specific probe p $\alpha$ 12H8 (tetramethyl-rodamine isothiocyanate TRITC; red signal) and 12p paint (FITC, yellow signal), and DAPI as counterstaining of the chromosomes: No 12p- or centromere-derived sequences are present on the cytogenetically identified i(12p) (arrow), whereas 12p and centromere sequences are hidden in the cytogeneti-

of the fluorescent centromeric region of the i(12p) as compared with the normal chromosomes 12 detected in metaphase spreads of the cell lines was not observed consistently on interphase nuclei hybridized in the same experiment. Therefore, FISH with only a centromeric region-specific probe is not sufficient to screen for the presence of one or more i(12p)s in interphase nuclei. This conclusion is strengthened by the fact that in two cell lines (2102Ep and H12.1) a reduced size of the hybridizing centromeric regions was also observed in derivatives of chromosome 12 other than i(12p). Therefore, the finding of i(12p) in pediatric germ cell tumors, as recently reported [13], must be verified. The double FISH approach used in this study might be informative.

As compared with conventional karyotyping, additional chromosome 12-derived sequences were detected with FISH in H12.1 and S2. All cell lines contained a relative overrepresentation of the centromeric region and short arm sequences of chromosome 12 as compared with their modal chromosome constitution (hypotriploid). CGH showed overrepresentation of 12p sequences only in the cell lines with more than one extra copy of the complete short arm of chromosome 12 [those with at least one i(12p)]. The ratio of the 12p-derived signals to those reflecting the centromeric regions indicates the presence (>1.0) or absence ( $\leq$ 1.0) of one or more i(12p)s. This ratio might be useful to study tumors for the presence of the i(12p), using interphase cytogenetics. This is currently under investigation. To visualize the presence or absence of i(12p) on interphase nuclei, a 12p-specific probe mapping closer to the centromere than YAC#5 would be more informative. Double FISH proved a suitable method for the detection of 12p overrepresentation in general and of i(12p) in particular on metaphase spreads and interphase nuclei of TGCT-derived cell lines. Currently, we are determining the critical region of overrepresentation of the short arm of chromosome 12 in TGCT using a combination of cytogenetic and FISH analysis. Because CGH confirmed the cytogenetic findings on chromosome 12 of the cell lines included in this study, and because of the recent report of the detection of amplification of a restricted region of the short arm of chromosome 12 in a metastasis of a SE [23], as well as in primary TGCTs [43], we will also use this technique in the analysis of the 12p aberrations in primary TGCT. This combined approach will finally result in the identification of the chromosome region from which the candidate gene or genes causing development of this cancer can be isolated.

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 cally identified der(4)(4;12)(p13;q11) (lower right). (B) Double FISH with p $\alpha$ 12H8 (TRITC) and yeast artificial chromosome (YAC#5) (FITC) on a metaphase spread of S2; there was no hybridization of YAC#5 with the cytogenetically identified der(4)t(4;12)(p13;q11) (arrow). (C) FISH with p $\alpha$ 12H8 (TRITC) and YAC#5 (FITC) on a metaphase spread of H12.1 showing centromeric region in the add(18)(p11.1) (arrow). (D) Original photograph of a single FISH on a metaphase spread of NT2 with p $\alpha$ 12H8 (FITC) and propidium iodide as counterstaining of the chromosomes showing enlarged centromeric hybridizing regions of the i(12p)s (arrows) as compared with their normal counterparts. (E) Original photograph of a double FISH with p $\alpha$ 12H8 (TRITC) and YAC#5 (FITC) on an interphase nucleus of NT2 showing three normal chromosomes 12 (one centromeric and one YAC signal) and two i(12p)s (one centromeric and two YAC signals), without size differences of the TRITC-signals. (F) Overrepresentation of the short arm of chromosome 12 in NT2, as detected by comparative genomic hybridization.

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