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Demonstration of the Genuine Iso-12p Character of the Standard Marker Chromosome of Testicular Germ Cell Tumors and Identification of Further Chromosome 12 Aberrations by Competitive In Situ Hybridization

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Summary

The recently developed competitive in situ hybridization (CISH) strategy was applied to the analysis of chromosome 12 aberrations in testicular germ cell tumors (TGCTs). DNAs from two rodent-human somatic cell hybrids, containing either a normal chromosome 12 or the p arm of chromosome 12 as their unique human material, were used as probes. Our results demonstrate a genuine iso-12p character of the standard marker chromosome in TGCTs. Moreover, variant markers were identified representing translocation products that also involve chromosome 12.

Introduction

Testicular germ cell tumors (TGCTs) was characterized by a highly specific marker chromosome which, according to its morphological appearance, has been described as an iso-12p chromosome (Atkin and Baker 1983). The same marker chromosome has been detected in dysgerminomas (ovarian germ cell tumors [Atkin and Baker 1987; Jenkins and McCartney 1987]), and in some extragonadal germ cell tumors (Chaganti et al. 1989; Dal Cin et al. 1989; T. W. A. de Bruin, unpublished data). The frequent and consistent occurrence of the iso-12p chromosome may be indicative of an important role in the development of these various germ cell tumors. Iso-12p negative TGCTs have also been described, but in these cases other chromosome 12 aberrations are often found (Gibas et al. 1986; Castedo et al. 1988). Since the iso-12p marker chromosome has so far been defined by morphological criteria only, the nature of this anomaly still needs verification.

Here we describe the application of competitive in situ hybridization (CISH) techniques (Kievits et al. 1990) to the identification of chromosome 12 aberrations in primary tumors and TGCT-derived cell lines. The probes used have recently become available and consist of DNAs from two rodent-human somatic cell hybrids, one containing an entire chromosome 12 (constructed as described by Warburton et al [1990]) and another one containing a Pallister-Killian-derived isochromosome 12p (Zhang et al. 1989) as the only human material present. Our results indicate that a genuine iso-12p chromosome as well as variant markers with involvement of chromosome 12p are present in the tumor and tumor-derived cell lines.

Material and Methods

Cells and Preparation of Metaphase Spreads

The Chinese hamster-human somatic hybrid cell line PK-89-12, containing a normal human chromosome 12, was constructed as described by Warburton et al. (1990). The mouse-human somatic hybrid cell line M28, containing the short arm of chromosome

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12 (as an isochromosome 12p derived from a patient with Pallister-Killian syndrome) as the only human chromosome, was developed by Zhang et al. (1989). The primary tumors used in this study included a primary testicular nonseminomatous germ cell tumor and a residual mature teratoma following chemotherapy. The N Tera-2 clone D1 (NT2/D1) and Scha-1 cell lines were used as typical representatives of two distinct human TGCTs (embryonal carcinomas [Thompson et al. 1984; Andrews et al. 1987]).

Cells were cultured in F10 or RPMI-1640 medium, supplemented with antibiotics, glutamine, and 10%–15% FCS. Metaphase spreads from NT2/D1 cells, Scha-1 cells, primary tumors, and human blood lymphocytes (derived from healthy donors) were prepared according to standard protocols (Vos et al. 1990). Chromosome preparations were stored in 70% ethanol at 4°C until use. G-banding was carried out according to standard procedures.

Probes and Biotin Labeling

DNA from rodent-human hybrid cell lines PK-89-12 and M28 was isolated and purified as described previously (Geurts van Kessel et al. 1989). The DNAs were sonicated to a fragment length of about 300-500 bp. Subsequently, they were used as probes. Biotinylation of these probes was performed by standard nick-translation using Bio-11-dUTP (Sigma) or Bio-16-dUTP (Boehringer). After purification, DNA was dissolved at a concentration of 10 ng (PK-89-12) or 100 ng (M28) per microliter of hybridization mixture containing 50% (v/v) deionized formamide, 2 × SSC (final pH 7.0), 10% (w/v) dextrane sulfate, 1% (v/v) Tween-20, sonicated herring sperm DNA (5 μg/μl) as carrier DNA, and an excess sonicated total human DNA (see below) as competitor DNA.

Prehybridization and Hybridization

Competitive in situ hybridization (CISH) experiments were carried out according to protocols developed by Kievits et al. (1990) with some slight modifications. Prehybridization (partial reannealing) of PK-89-12 or M28 was carried out in the presence of an excess total human DNA (500 ng/μl or 1 μg/μl hybridization mixture, giving competitor:probe ratios of 50:1 or 10:1) at 37°C for 2 h or 24 h, respectively. Before hybridization, the chromosome slides were washed in PBS, dehydrated, airdried, and finally prewarmed at 70°C. Denaturation of the slides was achieved by immersing them in 70% (v/v) formamide, 2 × SSC (final pH 7.0) at 70°C for 3-5 min. The slides were taken out and, subsequently, the partially reannealed probe mixture was added to the slides (10 μl per 18 × 18-mm coverslip). Hybridizations were performed in a moist chamber at 37°C for 3 d.

Immunocytochemical Detection

After hybridization, the slides were washed three times in 50% (v/v) formamide, 2 × SSC (final pH 7.0) and 2 × SSC (pH 7.0) at 42°C for 5 min and once in 4 × SSC, 0.05% (v/v) Tween-20 (final pH 7.0) at room temperature for 3 min. Then the slides were incubated with 5% nonfat dry milk (Protifar, Nutricia) in 4 × SSC, 0.05% Tween-20 at room temperature for 5 min, as described by Kievits et al. (1990). Fluorescent staining of the hybridized probe was accomplished by alternate incubations with fluorescein-avidin DCS and biotinylated goat anti-avidin antibody (Vector Laboratories), until two layers of avidin were applied, essentially as described by Pinkel et al. (1986). Finally, the slides were mounted in antifade medium, supplemented with 1,4-diazobicyclo-(2,2,2)-octane (DABCO; Sigma) and 4,6-diamino-2-phenylindole (DAPI; Sigma) or propidium iodide (PI; Kodak) for counterstaining.

Results and Discussion

In the cell hybrids PK-89-12 and M28, whose DNA was used as a probe, the human chromosome (chromosome 12 in PK-89-12) and isochromosome 12p in M28) carried histidinol or neomycin resistance genes, respectively, so that they could be selected for during culture (Zhang et al. 1989; Warburton et al. 1990). The presence of the chromosomes in the hybrid cell lines was visualized by fluorescence in situ hybridization with total human DNA as a probe on hybrid metaphase spreads as described by Pinkel et al. (1986). In these lines one copy (PK-89-12) or two to three copies (M28) of the human chromosome were found to be present per cell (Geurts van Kessel et al., in press). In addition, the origin of the human chromosomes in the hybrid cell lines was confirmed by competitive in situ hybridization (CISH; see Kievits et al. 1990) using hybrid cell DNA as a probe on normal human blood lymphocyte chromosomes (unpublished results).

When PK-89-12 DNA was hybridized onto metaphase spreads of the first tumor, a primary testicular nonseminomatous germ cell tumor, the probe revealed the presence of three copies of chromosome 12 and four copies of a marker chromosome resembling an

iso-12p chromosome both in size and morphology (fig. 1*A*). The resulting fluorescent staining of both types of chromosomes appeared to be somewhat less intense near the centromeres. This may be explained by the presence in these regions of highly repeated sequences, which are competed for during prehybridization steps (Kievits et al. 1990). Under the stringent conditions used here, a high signal:noise ratio was achieved, which underlines the specific character of this probe.

In metaphase spreads of the second tumor, a residual mature teratoma following chemotherapy, again three copies of chromosome 12 and one marker chromosome, morphologically resembling an iso-12p, could be detected (fig. 1*B*). In addition, a second marker chromosome—a translocation product between a positively staining part of chromosome 12 (probably, but not unambiguously 12p) and another negatively staining and as yet unknown chromosome—was observed.

These results are in agreement with the occurrence of several copies of chromosome 12 and, in addition, one or more iso-12p marker chromosomes in TGCTs (Atkin and Baker 1983). Furthermore, these results demonstrate both the high specificity of the PK-89-12 probe in visualizing chromosome 12 sequences in metaphase spreads and its value in monitoring the occurrence of chromosome 12 aberrations.

Because no further material was available from the primary tumors tested above, M28 DNA was used as a probe on two cell lines, NT2/D1 and Scha-1, which are considered to be typical representatives of TGCTs, to assess the short-arm origin of the chromosome 12 sequences in TGCTs. NT2/D1 has previously been reported to contain, besides three copies of chromosome 12, a germ cell tumor-specific iso-12p chromosome (Thompson et al. 1984; Geurts van Kessel et al. 1989). CISH with M28 DNA on metaphase spreads of this cell line indeed showed the presence of three chromosomes with positively staining short arms; they most likely represent copies of chromosome 12 (fig. 1*C*). In addition, two marker chromosomes twice as large as 12p stained 12p positive. From this result we conclude that the earlier cytogenetically defined germ-cell tumor–specific iso-12p chromosome indeed is a true iso-12p, occurring in TGCTs, whose genuine character can be demonstrated by CISH using M28 DNA as a probe. Again, as found for the PK-89-12 probe, the specific fluorescent staining of the chromosomal arms after CISH with M28 DNA appears to be less dense in the centromeric and more intense in the telomeric regions. In some NT2/D1 metaphases a tiny nonhybridizing chromosomal fragment seemed to be translocated onto one of the two original iso-12p chromosomes (not shown). The second TGCT cell line used, Scha-1, does not contain a characteristic iso-12p marker chromosome according to cytogenetic analysis (Vos et al. 1990). Instead, these cells contain a 12p-derived marker chromosome that in size and morphology is reminiscent of an iso-12p chromosome and, in addition, another larger marker presumably containing chromosome 12 material. These observations were confirmed by CISH with PK-89-12 (not shown). CISH results with M28 DNA (see fig. 1*D*) indicate that the smaller marker chromosome indeed is a translocation chromosome, which is composed of a p arm of chromosome 12 translocated onto another, unknown chromosomal fragment. This result definitively excludes the smaller marker from being an iso-12p chromosome. The larger marker chromosome clearly contains 12p material, again translocated onto/into another chromosomal fragment: a 12p-positive fluorescent staining region in the central part of the marker chromosome, twice as large as seen on the normal 12p arm, was visualized (fig. 1, *bottom right*). By combining CISH data with G-banding data (see fig. 2) this marker can be interpreted as follows: an abnormal chromosome 12, with a double amount of 12p and a less-than-normal amount of 12q sequences, translocated onto another unidentified chromosomal fragment. From these results, we conclude that, in Scha-1 cells, chromosome 12 has undergone complex rearrangements involving both p and q arms. No typical iso-12p chromosome is present in these cells.

Taken together, our results demonstrate that the marker chromosome of TGCTs has a genuine iso-12p character, identifiable by CISH using hybrid (PK-89-12 and M28) DNA. Moreover, our data confirm previous observations indicating that rearrangement of chromosome 12, particularly 12p, may play an important role in TGCT development. Although a typical iso-12p chromosome is frequently observed and, via this study, easily identified as such, rearrangement of chromosome 12 does not necessarily have to result in this particular marker. These observations may indicate that in those cases in which no iso-12p chromosome is observed, a chromosome 12 (especially 12p) reshuffling may still have taken place, which can be detected by in situ hybridization techniques as described here. Such variant cases may be of particular value in defining the region(s) in chromosome 12 that are specifically involved in TGCT development. Addi-

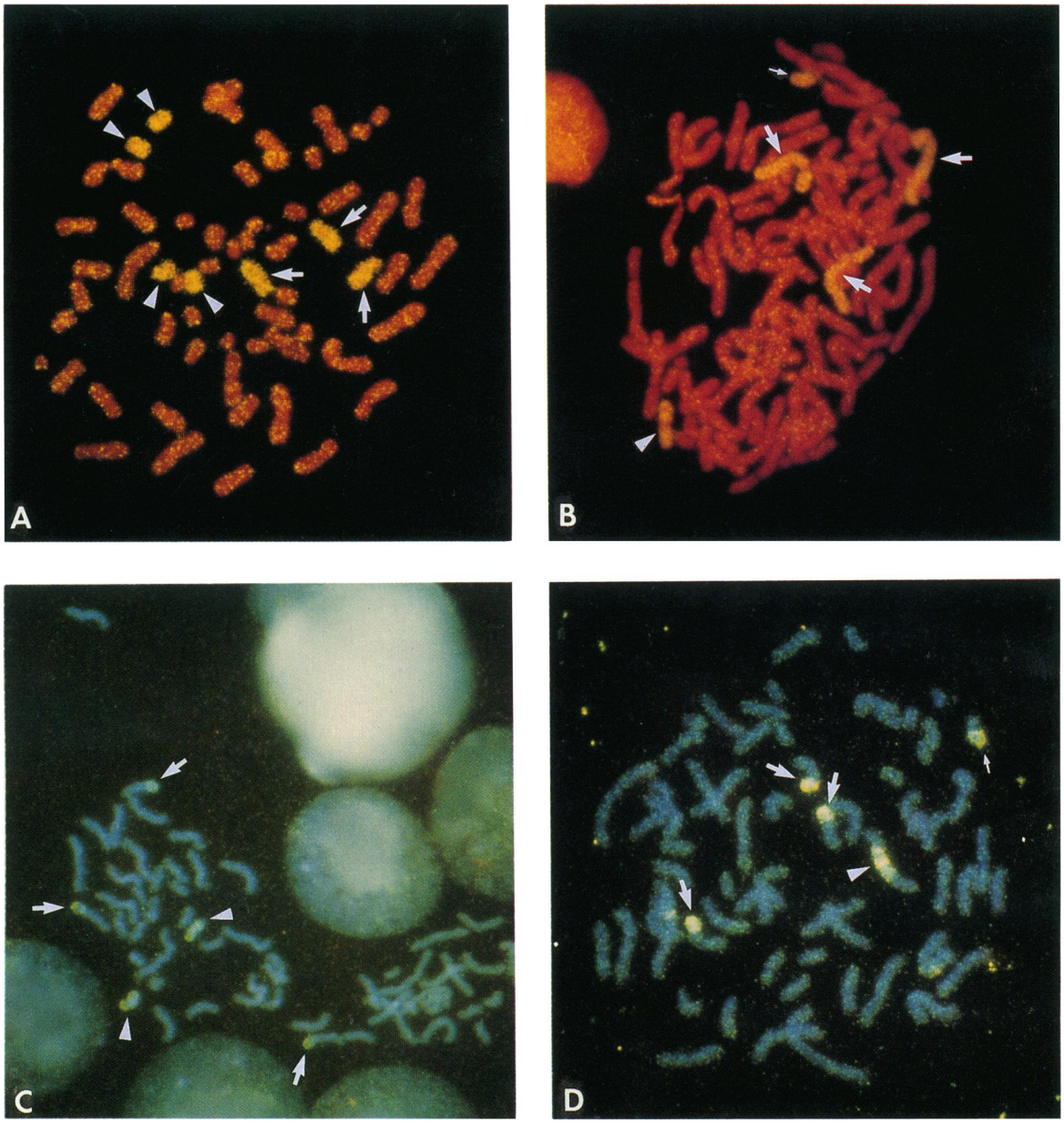

Figure 1 Results from CISH experiments using biotin-labeled DNA from two monochromosomal rodent-human somatic cell hybrids, specific for chromosome 12 (PK-89-12) and chromosome 12p (M28), as probes on metaphase spreads of TGCTs and TGCT-derived cell lines. Chromosomal regions hybridized with either of these probes were visualized with the fluorochrome FITC, resulting in a green/yellow fluorescent staining of these regions. Counterstaining of the chromosomes was performed with PI (*A* and *B*) or DAPI (*C* and *D*), resulting in a red or blue staining, respectively. *A,* In situ hybridization of PK-89-12 DNA to a metaphase spread of a TGCT (a primary nonseminomatous TGCT), showing complete fluorescent staining of three copies of chromosome 12 (*arrows*) and four markers most likely representing iso-12p marker chromosomes (*arrowheads*). *B,* In situ hybridization of PK-89-12 DNA to a preparation of a TGCT (a residual mature teratoma following chemotherapy). The metaphase spread displays three copies of chromosome 12 (*arrows*) and two markers: one marker resembles an iso-12p chromosome (*arrowhead*) and the other marker is a translocation chromosome in which, probably, a 12p arm is involved (*small arrow*). *C,* In situ hybridization of M28 DNA to a preparation of a TGCT-derived cell line (NT2/D1). The metaphase spread most likely displays three copies of chromosome 12, identifiable by their positively staining short arms (*arrows*). Furthermore, two iso-12p marker chromosomes are clearly recognizable (*arrowheads*). *D,* In situ hybridization of M28 DNA to a metaphase spread of a TGCT-derived cell line (Scha-1). Three copies, most likely of chromosome 12, identifiable by their positively staining short arms (*arrows*), and two marker chromosomes—both translocation chromosomes—can easily be distinguished. The small, metacentric translocation chromosome (*small arrow*) contains only one positively staining arm. The large translocation chromosome possesses a centrally located, positively staining, region (*arrowhead*).

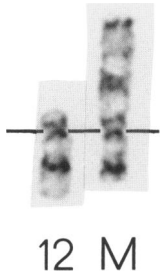

Figure 2 Giemsa-banded partial karyotype from the TGCT-derived cell line Scha-1 showing a normal chromosome 12 (*left*) and the large marker (M; *right*) in which chromosome 12 is involved (as described in the text). The 12p positively staining region, as found in fig. 1*D*, comprises an area ranging from the centromere to about halfway up the upper chromosomal arm.

tional probes derived from, e.g., the centromeric region of chromosome 12 as recently isolated by Looijenga et al. (1990) and repeated and/or single-copy fragments derived from 12p or 12q may prove to be of particular help in this respect. Further experiments with such probes are currently in progress.

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