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Original Paper

Quantification of Additional Short Arms of Chromosome 12 in Germ Cell Tumours Using the Polymerase Chain Reaction

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Male germ cell tumours are characterised by the over-representation of 12p sequences, most often in the form of isochromosome i(12p). This study describes the development of a quantitative detection system for additional copies of 12p employing the polymerase chain reaction (PCR). The validity of this method was assessed on two i(12p) containing tumour cell lines in which the number of i(12p) was determined by fluorescence *in situ* hybridisation. Fourteen primary male germ cell tumours were analysed using the PCR-based method. While 3/8 seminomatous germ cell cancers did not contain any additional 12p, all 6 non-seminomatous tumours did and the severity of the disease correlated with the respective copy number. The ease of the PCR-based method makes it possible for the quantification of additional 12p to become a routine diagnostic and prognostic tool for testicular germ cell tumours, thereby helping to define the role of the i(12p) anomality in larger retrospective studies. © **1997 Elsevier Science Ltd.**

Key words: carcinoma of uncertain histology, germ cell tumour, isochromosome 12p, molecular diagnosis, polymerase chain reaction

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INTRODUCTION

GERM CELL tumours (GCTs) are the most frequent malignancies of young men between 15 and 35 years of age [1]. GCTs contribute to 1% of all male neoplasms and their incidence in European countries is steadily increasing [2, 3]. A variety of different chromosomal alterations leading to peritriploid chromosomal sets have been recognised in male GCTs. The most common and highly indicative change is the formation of an isochromosome of the p-arm of chromosome 12 [4, 5]. More than 80% of all male GCTs display one or more copies of isochromosome 12 [i(12p)] [6–14].

The definition of different risk groups is a prerequisite in the treatment of male GCTs to either prevent 'over-treatment' or to treat appropriately patients of high-risk groups as soon as possible. Thus, several prognostic factors such as tumour volume, α-fetoprotein levels in the serum and the degree and pattern of metastatic expansion are often determined [15, 16]. Over-representation of 12p sequence in the form of isochromosome i(12p) may represent another important prognostic factor since high i(12p) copy numbers are correlated with an unfavourable clinical course of GCT patients [17]. Furthermore, the presence of i(12p) in midline tumours of uncertain histogenesis may predict tumour sensitivity to *cis*-platinum/etoposide chemotherapy [18].

Use of the i(12p) copy number as a diagnostic and prognostic option has so far been hampered by technical difficulties of i(12p) detection and quantification. Conventional cytogenetic studies are restricted to cells growing in short-term culture, while the *in situ* hybridisation technique allows the detection of i(12p) in primary tumour material, but is laborious and technically elaborate [19, 20]. To circumvent these difficulties, we established a polymerase chain reaction

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(PCR) based method which allows the quantification of i(12p) or otherwise over-represented 12p sequences in small amounts of primary human GCT material. We subsequently utilised this method to study the relationship between 12p copy number and lethality in a group of 14 male patients with different types of GCTs.

MATERIALS AND METHODS

Cell lines and tumour samples

The previously described cell line H12.1 and the cell line H32 [22] were derived from male GCTs. Primary tumour samples were obtained from 14 previously untreated male patients who had undergone resection. Tumour samples were frozen in liquid nitrogen and stored at −80°C. Histopathological examinations were done in paraffin-embedded serial sections of the primary tumour.

DNA cloning

For the cloning of a 12q specific sequence, a 366 bp fragment was isolated from pYNH15 [23] and inserted into Bluescript KS$^+$ (Stratagene, Heidelberg). An unrelated 117 bp fragment was then cloned into the internal *Eco*RI site of the 366 bp *Pst*I insert, thereby generating pKS-H15. For the cloning of a 12p specific sequence, PCR was performed with the universal primer (Promega) and the SW-delta oligodeoxynucleotide (see below) utilising pSW11-1, which contains human *C-KI-RAS-2* cDNA [24], as a template. SW-delta hybridises to the end of *C-KI-RAS-2* exon 3 (sequence in italics) and is complementary to an internal sequence of the adjacent intron (sequence in bold-type). The resulting PCR product was digested with *Pst*I and *Hind*III, blunted with T4-DNA polymerase and cloned into pKS-H15. Thereby, the pH15/SW-standard was generated.

SW-delta oligodeoxynucleotide:

5′-GACCGAAGC **TTCCTAGTATAGCATAATTGAGAG***CTGTCTTGTCTTTGCTGATGT*

Competitive PCR

Genomic DNA used as a template was prepared according to a standard protocol [25]. A PCR reaction mix contained 100–400 ng mechanically sheared genomic DNA, 0.5 μM each of the four oligodeoxynucleotides listed below, 200 μM dATP/dCTP/dGTP/dTTP (including 1 mCi [α-^{32}P]dATP), 2 units Taq DNA polymerase (Promega) and 1 × PCR buffer (10 mM Tris-HCl pH 8.3, 2 mM $MgCl_2$, 50 mM KCl, 0.01% (wt/vol) gelatine) in a volume of 45 μl. 5 μl of serial 1:1 dilutions of *Eco*RV linearised pH15/SW standard (range: 20 pg to 0.04 pg) were added using aerosol-resistant pipetting tips and the reaction mix was overlaid with 50 μl paraffin oil. PCR was then performed using an initial denaturation step (94°C, 3 min), 5 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and extension 72°C, 2 min), and a further 25 cycles with a reduced denaturation temperature (92°C). 40 μl of each PCR sample were then subjected to electrophoresis on a 6% acrylamide gel. After staining with ethidium bromide PCR products were excised from the gel on a UV transilluminator and incorporation of radioactivity was measured by Cerenkov counting.

Oligodeoxynucleotides used:

SW-sense:
5′-AGAGTTAAGGACTCTGAAGATGTA

SW-antisense:
5′-TTCCTAGTATAGCATAATTGAGAG

H15-sense:
5′-GGACAAAACCCTCAAACACATAGA

H15-antisense:
5′-CAGCCAAGAGAGTGTTCTTTTTCT

Karyotypic analysis

Cells were cultured at 37°C in an atmosphere containing 5% CO_2 [22]. Metaphase spreads were prepared according to standard procedures and fixed with methanol–acetic acid. Air-dried preparations were banded using pancreatin [26].

RESULTS

In order to determine the number of additional 12p sequences by competitive PCR [27, 28], we utilised two primer pairs. The SW-sense/SW-antisense primer pair amplifies a sequence on the p-arm and the H15-sense/H15-antisense primer pair amplifies a sequence on the q-arm of human chromosome 12 (Figure 1). In addition, these primer pairs can also amplify a shortened version of the genomic 12p sequence ($p_{standard}$) and an elongated version of the genomic 12q sequence ($q_{standard}$) when employing pH15/SW-standard as a template (Figure 1). By using a serial dilution series of the pH15/SW-standard plasmid and a constant amount of genomic DNA for PCR, it is possible to determine those dilution steps (p* and q*) at which the p- and q-genomic products are equimolar to the respective p- and q-standard products. A representative determination of p* and q* with genomic DNA derived from non-GCT cells ('normal') is shown in Figure 2(a). The difference p*–q* was independent of the source of DNA (blood, placenta, osteosarcoma, rhabdomyosarcoma) and constant within a certain range of genomic DNA (100–400 ng) when PCRs were performed in parallel, yet varied from −0.7 to −1.3 in consecutive PCRs. The p*–q* value is essential for quantification of the 12p copy number. Since this value varied in consecutive PCRs, it was always determined for 'normal' and GCT-derived genomic DNA in parallel. Furthermore, all measurements were done in duplicate or triplicate.

The established GCT cell lines H12.1 (Figure 2(b)) and H32 showed an increase in the difference (Table 1) p*–q*, as expected due to the presence of i(12p) in these cell lines [22].

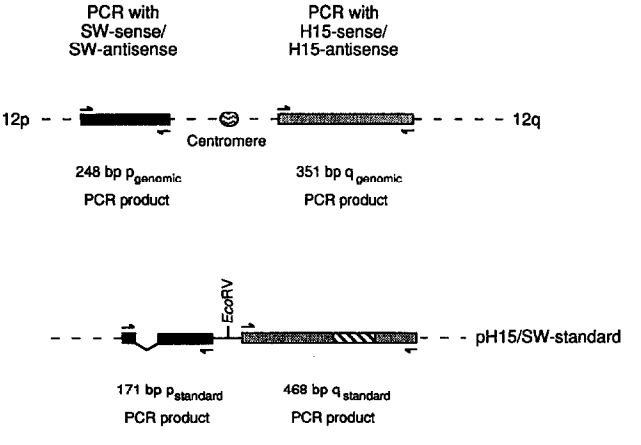

Figure 1. Schematic presentation of PCR products derived from chromosome 12 or pH 15/SW-standard when utilising jointly the SW-sense/SW-antisense and H15-sense/H15-antisense prime pairs.

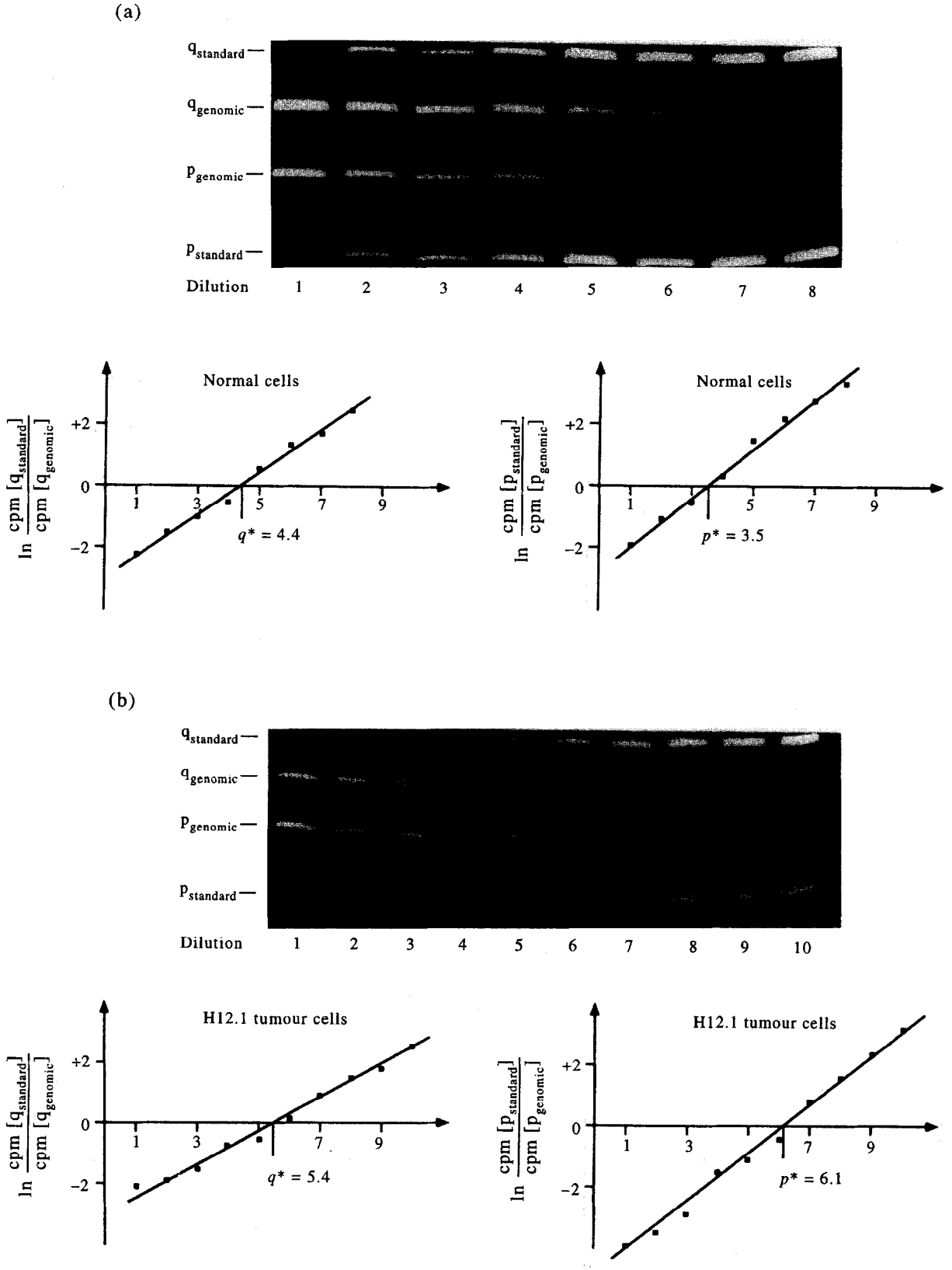

Figure 2. Competitive PCR wth normal cells (a) and H12.1 tumour cells (b) serial 1:1 dilutions of the pH15/SW-standard plasmid (No. 1 indicates the lowest concentration) were utilised with a constant amount of genomic DNA in the polymerase chain reaction. PCR products were visualised by ethidium bromide staining (see agarose gels). After measuring incorporation of radioactivity in the PCR products, those dilution steps at which genomic and standard products were equimolar (p* and q*) were determined by the intersection points of the regression lines with the abscissa (see line graphs).

Table 1. i(12p) copy number in established GCT cell lines

	$p^{*tumour}-q^{*tumour}$	$p^{*normal}-q^{*normal}$	i(12p) copies*
GCT cell line H12.1	0.7	−0.9	2.0
GCT cell line H32	0.5	−1.0	1.8

*Calculation of i(12p) copy number: $n = -1 + 2(p^{*tumour}-q^{*tumour})-(p^{*normal}-q^{*normal})$.

We could calculate an i(12p) copy number of 2.0 for H12.1 and 1.8 for H32. This takes into account that the number of additional p-arms is actually double since i(12p) is a fusion of two p-arms [29].

In order to validate these results, karyotyping of three independent H12.1 cell clones (clones A, B and C) was performed. Clones A and B revealed two copies of i(12p), as demonstrated by B clone in Figure 3 and clone C three copies of i(12p). Furthermore, while clones B and C displayed two copies of chromosome 12, clone A had three copies of chromosome 12, the latter in accordance with published data [33]. Thus, karyotyping of the H12.1 cell line suggested a mean number of 2.3 normal chromosome 12 and an average of 2.3 copies of i(12p), which fairly well matches the measurement of two copies of i12p with our PCR-based method. In addition, fluorescence *in situ* hybridisation (FISH) was performed with the H12.1 cell line. Two different DNA probes were employed for staining: pα12H8, which is a centromeric region probe, and YAC#5, which is specific for 12p11.2–p12.1 4.1 or 5.2 signals for 12p were detected (Table 2), which would indicate 1.8–2.9 copies of i(12p) considering the presence of an average of 2.3 normal chromosomes 12.

In the H32 cell line, karyotypic analysis of five metaphase spreads indicated two ($n = 2$) or three ($n = 3$) normal chromosomes 12 (average = 2.6 copies) and one i(12p) in 80% of the cases (data not shown). However, FISH analysis revealed 4 or 5 signals for 12p (Table 2), which, assuming a mean number of 2.6 normal chromosomes 12, indicates the presence of 1.4–2.4 copies of i(12p). Thus, karyotyping and FISH analysis of the H32 cell line displayed a significant degree of variance to each other, which may be due to the fact that deletion of small i(12p) chromosomes in the karyotypic analysis can be hampered. Relative to the FISH analysis, our determination of 1.8 copies of i(12p) via competitive PCR is again in good agreement, indicating the validity of this novel method to measure the i(12p) copy number.

To investigate whether this system is able to determine additional copies of 12p in human primary tumours, 14 male GCTs of varying histology were analysed (Table 3). 3 out of 8 patients with seminomatous GCTs showed no detectable i(12p), while 5 tumours had copy numbers ranging from 0.75 to 3.25 copies (mean number 1.16; $n = 8$). In the non-seminomatous group, all 6 patients were i(12p) positive with a maximum of 3.75 copies (mean number

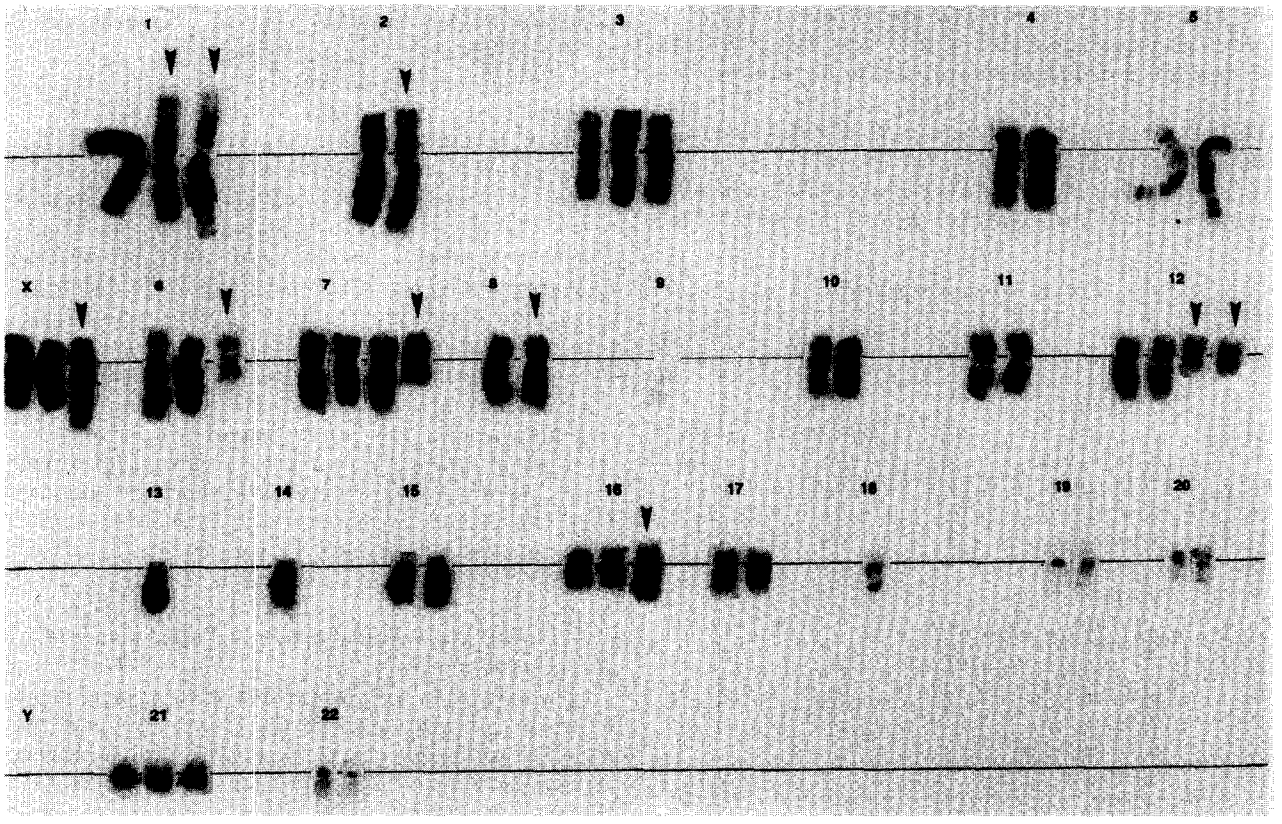

Figure 3. Representative karyotypic analysis of one H12.1 tumour cell.

Table 2. FISH analysis of the GCT cell lines

	MNS pα12H8 centromeric	MNS YAC#5 12p11.2–p12.1
GCT cell line H12.1	4.1	5.2
GCT cell line H32	4	5

MNS, mean number of spots per metaphase.

1.92; $n = 6$). Since most GCTs contain i(12p), we assumed that all additional 12p sequences were present in i(12p).

DISCUSSION

The aim of this study was to introduce a method which facilitates the detection and quantification of additional short arms of chromosome 12 in primary germ cell tumour material. We decided to use a PCR-based system, because PCR technology has become a well known and broadly used system in clinical oncology.

By comparing the copy numbers of a gene located on the short arm of chromosome 12 (*C-KI-RAS-2*) with a sequence located on the long arm (YNH15) of the same chromosome, we were able to determine the ratio between 12p- and 12q-specific genetic material. The correct quantification of the 12p- and 12q-specific sequences was accomplished by introducing two internal plasmid standards, which extended the technique to a competitive PCR approach [27].

Since this method is based on the use of the SW-sense and SW-antisense primers, which are complementary to the *C-KI-RAS-2* proto-oncogene located on chromosome 12p, a duplication of this gene or the existence of *RAS* pseudogenes might falsify the results, because in these cases the *RAS* gene copy number would be measured instead of chromosome 12p amplifications. The fact that the SW-antisense primer is complementary to an intron sequence prevents the detection of pseudogenes, e.g. the *C-KI-RAS-1* gene [24]. Although a duplication of *C-KI-RAS-2* would be indistinguishable from *i*(12p) formation with our assay system, amplification of *C-KI-RAS-2* in male GCTs arises primarily from additional copies of 12p [21, 30]. In those rare cases in which a selective duplication of the *C-KI-RAS-2* gene has occurred in male GCTs, amplification of another 12p gene and a respective external standard could improve this system. The use of primer oligonucleotides directed against the recently described smallest region of over-representation of 12p [34] could help to detect 12p amplifications which do not include more proximal regions of the chromosome. Please note that *C-KI-RAS-2* may thus not always be amplified by the generation of i(12p), consistent with the notion that its amplification is not the cause for the development of GCTs.

Table 3. Primary male GCTs

Patient	Histology	Stage	Status*	i(12p) copies†
14A	seminoma	I	ned	1.75
22A	seminoma	II C	toxic death	1.75
26A	seminoma	I	ned	0
38A	seminoma	I	ned	3.25
44A	seminoma	II C	dod	1.75
79A	seminoma	I	ned	0.75
80A	seminoma	-	ned	0
81A	seminoma	-	ned	0
7A	mixed	III C	dod	3.75
8A	teratoma	III C	dod	3
13A	mixed	I	ned	1.25
95A	teratocarcinoma	I	ned	2
100A	seminoma/ teratoseminoma	II B	ned	0.75
101A	mixed	–	–	0.75

*Ned, no evidence of disease; dod, died of disease.
†Calculated as described in Table 1.

While amplification of the whole chromosome 12 would also increase the copy number of *C-KI-RAS-2*, as described here in the GCT cell lines H12.1 and H32, this would go undetected by this method due to standardisation to the 12q copy number. Thus, this method distinguishes between amplification of chromosome 12 and over-representation of 12p sequences, with only the latter being correlated with male GCTs.

The validity of this PCR-based method to quantitate the i(12p) copy number was assessed by comparing its results to those obtained by cytogenetic investigation and FISH analysis. In the case of the H12.1 cell line, both karyotyping and FISH analysis confirmed our results, and the FISH analysis of the H32 cell line was also in good agreement with our quantification of i(12p). Thus, the novel method described in this paper is another valid approach to measuring i(12p) copy numbers.

In addition to the described cell lines, we investigated a group of 14 GCTs to show that the technique is able to determine the i(12p) copy numbers in primary germ cell tumour material. DNA prepared from solid tumours is always contaminated by 'normal' DNA which could lead to falsified results. However, histopathological examinations demonstrated that all GCTs analysed contained more than 80% malignant cells (data not shown).

In the group of seminomatous germ cell tumours, we observed i(12p) copy numbers ranging from 0.75 to 3.25 in 5 out of 8 tumours; three tumours displayed no i(12p). These numbers are in accordance with previously published studies, showing that approximately 70% of the seminomatous GCTs investigated contain the i(12p) anomality [8]. The tumours negative for the i(12p) anomality are thought to display a different GCT subgroup [13]. Although the overall number of chromosomes in seminomatous germ cell tumours is increased (35), this is not excessively, and our PCR-based method to quantitate i(12p) is still capable of reliably detecting one i(12p) in cells containing 4–6 normal chromosomes 12. This argues against the assumption that measurement of no i(12p) in three of the analysed seminomatous GCTs is due to the detection limit of our competitive PCR method. In contrast, i(12p) was detected in all of the 6 non-seminomatous GCTs, with the maximum number being 3.75 copies. The mean numbers of i(12p) in the seminoma group (1.16; $n = 8$) and in the non-seminoma group (1.92; $n = 6$) agree fully with those reported before (1.2 and 2.0, respectively) [8].

Previous studies have shown that three or more copies of i(12p) in non-seminomatous GCTs are associated with a more severe clinical course, particularly with resistance to systemic treatment [17]. Thus we tried to correlate the i(12p) copy number to the clinical course of the patients (Table 3). Three patients died as a result of their disease, 2

of them having non-seminomatous GCTs. These 2 (patients 7A and 8A) had the highest number of i(12p) among the non-seminomatous GCT group. In contrast, increased i(12p) copy numbers did not correlate with lethality in the group of seminomatous GCTs. Thus introduction of our PCR system into clinical routine procedures would allow determination of the prognostic role of the i(12p) anomality in a larger group of patients.

The simplicity of the quantitative PCR method to measure additional 12p sequences should improve the detection of i(12p) in male GCTs. Direct cytogenetic studies are limited to cells growing in short-term culture, which non-specialised centres might have difficulties in performing. The DNA used as a template within the PCR process can be purified from almost any material, which opens the possibility of screening for the i(12p) anomality in tissue samples obtained during surgical procedures or in paraffin-embedded slides. In contrast to other cytogenetic methods, particularly the FISH technique, it is not dependent on experienced interpretation of the results obtained [18]. Quantification of the i(12p) copy number can be achieved by simple direct calculation, using the quantities of the different PCR products described herein.

In summary, this technique offers a reliable new possibility for detecting and quantitating additional 12p sequences in primary germ cell tumour material. In addition, it is relatively easy to perform and not restricted to cells growing in short-term culture. The possibility of being able to purify DNA from paraffin-embedded tumours allows the role of the i(12p) anomality to be evaluated in larger retrospective analysis.

Furthermore, this method could be of prophylactic value by detecting the precursors of GCTs, the carcinoma *in situ* cells [9, 31]. In addition, it might provide guidance for the treatment of patients with midline carcinomas of uncertain histology [32]. Detection of i(12p) in these cases will suggest selective treatment with chemotherapeutic drugs such as *cis*-platinum/etoposide [1, 18] and thereby provide a chance to cure such patients.

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