MOLECULAR CYTOGENETICS OF HUMAN TESTICULAR GERM CELL TUMORS

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Moleculaire cytogenetica van humane testiculaire kiemceltumoren

Proefschrift

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Cover: detail photograph of raku ceramics

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"Voor het kennen van de Weg,
Moeten we gewoon op Weg.
Je dingen doen,
Liefst met plezier,
Heel simpel op de Poeh-Manier.
Maar ga niet zoeken naar de Weg,
Want je zal zien, dan is hij weg"

Citaat uit: Tao van Poeh

Aan mijn ouders,
Leendert, Maarten en Anna

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Chapter 1

General introduction

1.1 Chromosomal aberrations in cancer

It was not until 1960, with the finding of the Philadelphia chromosome in chronic myelocytic leukemia (Nowell and Hungerford 1960), that the first specific chromosomal abnormality was definitively associated with human cancer. This in spite of the fact that already more than 40 years earlier cancerous growth was associated with an aberrant genome (Boveri 1914). The main reason for this delay was the lack of suitable tools for isolation and identification of human chromosomes.

After the development of specific staining methods in the early seventies, initially mainly non-solid tumors were cytogenetically analysed because their metaphases were more easily obtained. Subsequently with the development of more recent cytogenetic techniques, chromosomal analysis of solid tumors became more feasible. A wide variety of chromosomal aberrations are currently identified in human neoplasia (for review; Mitelman et al. 1997). These comprise numerical changes, resulting in gains or losses of whole chromosomes, structural changes as translocations, inversions, deletions, homogeneously stained regions (HSRs) and double minutes (DMs). An example of an abnormal chromosomal pattern as found in a solid tumor is shown in Figure 1.

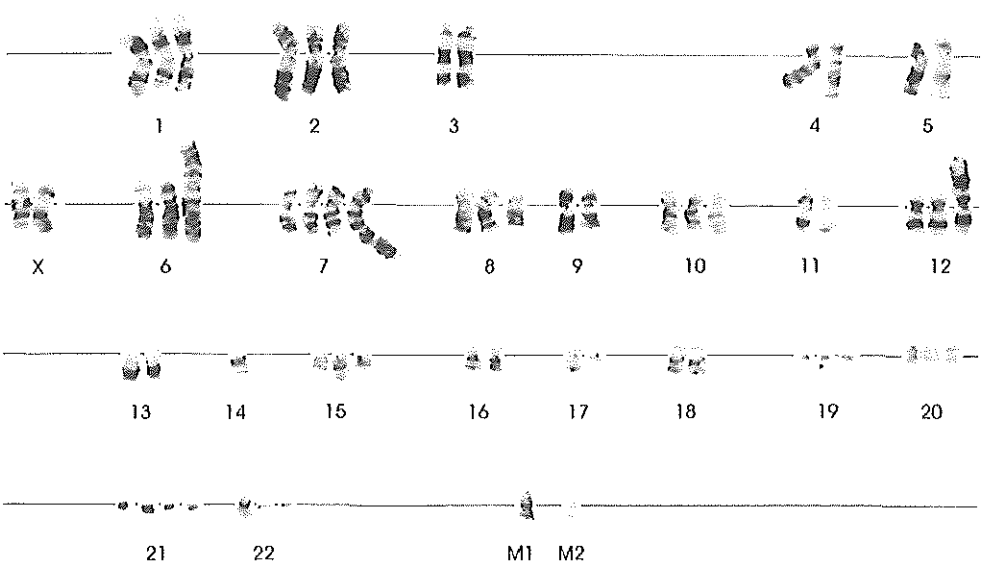

Fig. 1: Karyotype of a testicular seminoma showing various chromosomal aberrations: numerical (chromosomes 1,2,6,7,8,10,12,14,15,19,20,21,22,X and Y), structural (chromosomes 6,7,8,11 and 12) and two unidentified chromosomes (markers, M1 and M2).

While some aberrations can be inherited, the majority occurs *de novo*. They can either originate spontaneously (Bischoff et al. 1990) or under the influence of chemical or physical carcinogens (Li et al. 1997) or viruses (Akagi et al. 1989; Namba et al. 1996), or a combination of them. When these abnormalities are primary and causative, they can be specific for a certain type of cancer and may be useful for diagnostic and/or prognostic purpose (Karakousis et al. 1987; Sandberg 1988; Trent et al. 1989; Fletcher et al. 1991). However, most are secondary, related to the progression of cancer, and therefore less specific (Johansson et al. 1996).

It has become clear that most solid cancers are clinically manifest after undergoing several genomic aberrations, involving multiple (parts of) chromosomes. Therefore, it usually takes decades for an incipient tumor to accumulate the aberrations required for malignant growth. The first cancer of which the sequential genomic aberrations were elucidated was colon cancer (Vogelstein et al. 1988). Its carcinogenesis appears to be a multistep process, in which at least five (epi-)genetic changes are necessary for complete malignant transformation of normal colon epithelial cells.

Molecular changes in cancer

With the development of molecular techniques in the seventies, it became obvious that the chromosomal aberrations found in cancer, are indicators for smaller alterations at the DNA level. These alterations may influence various processes within a cell, e.g. DNA repair, apoptosis and cell cycle control (Pearson and Van der Luijt 1998; Ilyas et al. 1999; Pihan and Doxsey 1999). Three main categories of genes are involved in carcinogenesis: proto-oncogenes, tumor suppressor genes and DNA mismatch repair genes. Aberrations of these particular genes may influence tumor growth in different ways. Due to mutation, translocation or amplification proto-oncogenes can be activated (oncogenes) thereby stimulating tumor growth. A similar effect can be reached by inactivation of a tumor suppressor gene, either via physical loss, mutation or methylation. The DNA repair genes contribute in tumor development when they are mutated, which can lead to activation of proto-oncogenes and inactivation of tumor suppressor genes (Kinzler and Vogelstein 1997). In general, tumors with a defect in the repair system, characterised by microsatellite instability, are diploid (Fishel et al. 1993). Thusfar, only few mutations have been found in mismatch repair genes especially in nonpolyposis colorectal carcinoma (Leach et al 1993; Peltomäki et al. 1993). In contrast, numerous (proto-)onco- and tumor suppressor genes are involved in a wide range of human cancers (see for review http://www.biomedcomp.com and http://www.ncbi.nlm.nih.gov/ncicpap/CGAP). Some of these genes are frequently involved in different cancers. For instance, the tumor suppressor gene *P53* is inactivated in approximately 50% of solid malignancies including breast, lung and colon cancer. Among the proto-oncogenes, the *RAS* genes (K-, H-, and N-*RAS*), coding for regulatory proteins in signal transduction, are most frequently mutated in colon and pancreatic carcinomas and adenocarcinomas of the lung and ovary (Anderson et al. 1992).

In conclusion, activation (proto-oncogenes) and inactivation (tumor suppressor genes) of multiple genes play a role in the development of cancer, and are often associated with a variety of chromosomal abnormalities. Identification of these specific chromosomal anomalies is an important step in unravelling the molecular mechanisms of the pathogenesis of a particular type of cancer. Furthermore, these findings may have applications in early detection, diagnosis and prognosis of cancer (Jones and Fletcher 1999).

1.2 Methods for chromosomal analysis of cancer cells

A major breakthrough in the analysis of chromosomal aberrations was the introduction of the banding techniques in 1970 (Caspersson et al. 1970). It allowed the identification of all human chromosomes based on specific banding patterns and led to a fast development in the characterisation of specific chromosomal abnormalities in cancer. Most karyotypic data have been obtained from hematological malignancies (Mitelman 1991), from which metaphases are more easily obtained than from solid tumors. Therefore, the search for alternative cytogenetic methods, that circumvent the need of cells in mitosis, continued, and was beneficiated with the development of *in situ* hybridisation. Although this technique was already discovered in 1969 (Pardue and Gall 1969), it only became widely applied in the eighties, with the introduction of fluorescence detection methods, i.e. fluorescence *in situ* hybridisation (FISH) (Pinkel et al. 1986). More recent developments of FISH resulted in comparative genomic hybridisation (CGH) (Kallioniemi et al. 1992; Du Manoir et al. 1993) and 24-colour hybridisation, also known as multi-colour FISH and SKY (Schrock et al. 1996; Speicher et al. 1996). These developments have expanded the knowledge of chromosomal aberrations in solid tumors (see for review http://www.ncbi.nlm.nih.gov/CCAP/mitelsum.cgi). An overview of the cytogenetic methods and their application in tumor biology is shown in Table 1. Because FISH and CGH were applied in the studies described in Chapters 2-7 of this thesis, these methods will be discussed in more detail in the next paragraphs.

Fluorescence *in situ* hybridisation (FISH)

The radioactive *in situ* hybridisation approach, introduced by Gall and Pardue in 1969, was the basis for the development of several non-radioactive modifications. Because this technique is relatively fast and easy, FISH has become a widely used method for mapping, biomedical research and diagnostic applications (for review see Le Beau 1993; Fox et al. 1995; Siebert and Weber-Matthiesen 1997; Werner et al. 1997; Luke and Shepelsky 1998). Whereas, conventional cytogenetics was limited by the low number and often poor quality of metaphases, FISH, which can also be applied on interphase nuclei, allowed identification of genetic alterations in many previously unsolved and difficult cases. The technique is based on hybridisation of a single DNA strand (probe) to a single homologous stranded sequence (target), fixed on a slide. The probe can be labeled directly by incorporation of nucleotides conjugated

Table 1:

Overview of the applicability of the (molecular) cytogenetic methods used in this thesis for the identification of numerical and structural anomalies.

MATERIAL	METHODS	SUITABLE FOR IDENTIFICATION OF:	RESOLUTION:
Metaphase	karyotyping	numerical and (particular) structural anomalies based on chromosome-specific banding patterns.	>2 Mb
	FISH	numerical and the majority of structural anomalies visualised with specific probes. Mainly for refining of the characterization of previously detected re-arrangements.	>5 Kb
	SKY	numerical alterations, markers and interchromosomal rearrangements involving large chromosomal fragments; an overview of the genome	>2 Mb
Interphase (isolated nuclei/ tissue sections)	FISH	numerical anomalies and a minority of structural aberrations. Especially the detection of structural aberrations requires pre-knowledge of the re-arrangements and the need of spanning and flanking probes.	>5Kb
DNA	CGH	gains and losses (of parts) of all chromosomes; an overview.	≥2 Mb*

Abbreviations used: FISH = fluorescent *in situ* hybridisation; SKY = spectral karyotyping; CGH = comparative genomic hybridization.

* The resolution for gains is dependent on the combination of size of the region involved and copy number.

to fluorochromes or indirectly by incorporation of nucleotides conjugated to haptens, such as biotin or digoxigenin, and detected with fluorochrome conjugated molecules. The specific hybrid is visualised by fluorescence microscopy using appropriate filters. The probes can be of various sizes and complexity, recognizing repetitive sequences such as centromeric and telomeric regions or single copy sequences. A collection of probes covering an area, such as a chromosome or a part thereof, can be used as a "painting" probe (Yung 1996) (see Figure 2).

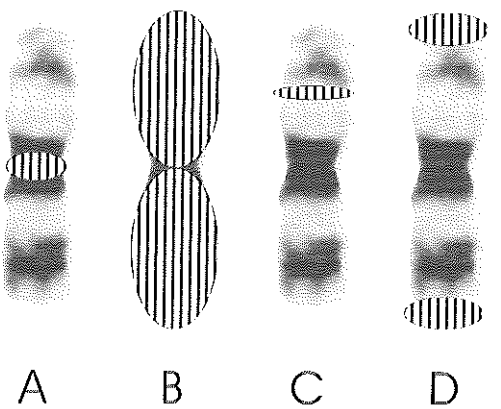

Fig. 2:
Schematic representation of different probes used for in situ hybridisation:
A: centromeric;
B: whole chromosome paint;
C: unique sequence and
D: telomeric.

For visualisation of a hybridised probe, fluorescein isothiocyanaat (FITC) is the fluorochrome most commonly used. For two colour (double) FISH and more colours simultaneously, fluorochromes as Texas Red, lissamine, rhodamine or its derivatives, AMCA or cascade blue are applied (Tanke 1999). Beyond that, multiplicity of FISH is significantly increased by the use of combinatorially or ratio-labeled probes (Nederlof et al. 1990; Ried et al. 1992). Simultaneous visualisation of all human chromosomes became possible by combinatorial labeling with five fluorochromes (Schrock et al. 1996; Speicher et al. 1996). Recently, combinatorial labeling was combined with ratio-labeling (Combined Binary Ratio labeling; COBRA) achieving a theoretical multiplicity of 48 with the same five fluorochromes (Tanke et al. 1999).

FISH is a powerful tool in cancer research. A major application is the assessment of numerical aberrations in interfase nuclei of tumor cells with centromeric probes (Barranco et al. 1998; Mendelin et al. 1999; Poetsch et al. 1999). Other examples are the precise mapping of deleted or amplified regions, (Bell et al. 1996; Elkahloun et al. 1996), identification of marker chromosomes (Boschman et al. 1993; Shi et al. 1993; Brandt et al. 1994; Arkesteijn et al. 1999) and translocations (Mancini et al. 1994; Janz et al. 1995; Shipley et al. 1996; Weber-Matthiesen et al. 1996).

Besides the use of FISH on metaphases and isolated nuclei, this technique can also be used on frozen tissue sections and formalin-fixed, paraffin-embedded material, allowing

analysis of chromosomal anomalies in cells within their histological context (Schofield and Fletcher 1992; Werner et al. 1997). The value of this method can be enhanced by its combination with immunohistochemistry (Weber-Matthiesen et al. 1993; Nolte et al. 1995).

The estimated lower detection limit of FISH lies between 1-5 kb of target DNA (Lawrence et al. 1988). However, targets of 10-40 kb are more suited for this technique. Several attempts have been made to improve the sensitivity either by amplification of the target, e.g. primed *in situ* (PRINS) polymerase chain reaction (PCR) or amplification of the signal *in situ* by, e.g. the CARD signal amplification method. The latter technique seems the most promising and has resulted in a significant increase in the signal intensity for the detection of both repetitive sequences as well as single copy genes (Raap et al. 1995, for review; Speel et al. 1999). The CARD amplification method is based on the deposition of a large number of haptenized tyramide molecules by peroxidase activity at the site of hybridisation. Visualisation of deposited tyramides can be performed either directly by the use of fluorochrome-labeled tyramides, or indirectly if biotin, digoxigenin or di- or trinitrophenyl conjugated to tyramide are used, and detected by fluorochrome-labeled molecules (Speel et al. 1995). With this method for signal-amplification the intensity of the FISH signal could be improved in the range of two- to 100-fold. Furthermore, it improves the detection of weak signals of cosmids in tumor material (this thesis) and allows the detection of repetitive and single-copy DNA sequences (up to the level of 1-5 kb) in cell preparations. However, in spite of the higher signal intensities generated by this detection system, it remains to be proven if it allows the visualisation of small targets which cannot be detected by standard methods.

Comparative genomic hybridisation (CGH)

Although FISH is a powerful technique, in contrast with classical karyotyping, it investigates a limited number of targets per experiment, and therefore the method requires previous knowledge on the areas to be investigated. This information is especially scarce for solid tumors. An alternative approach became available with the technique of CGH (Kallioniemi et al. 1992; Du Manoir et al. 1993). In a single experiment, CGH provides a global overview of relative chromosomal gains and losses compared to the average ploidy. Briefly, differentially labeled test (green) and reference DNA (red) are hybridised simultaneously to normal metaphase chromosomes. The ratio between the normalised intensities of the fluorescence signals, reflects differences in the amount of tumor DNA compared with normal DNA per chromosomal region. Chromosomal regions with an increased green/red fluorescence ratio represent DNA gains, while a reduced ratio is a consequence of loss. The average ratio is calculated for the entire length of each chromosome using a digital image analysis system, and plotted along ideograms of the corresponding chromosomes (see Figure 3). The potential of CGH has been significantly increased with the use of tissue microdissection followed by PCR amplification of DNA. This allows analysis of specific tissue areas or even individual cells (Speicher et al. 1993; Speicher et al. 1995; Wiltshire et al. 1995; Klein et al. 1999).

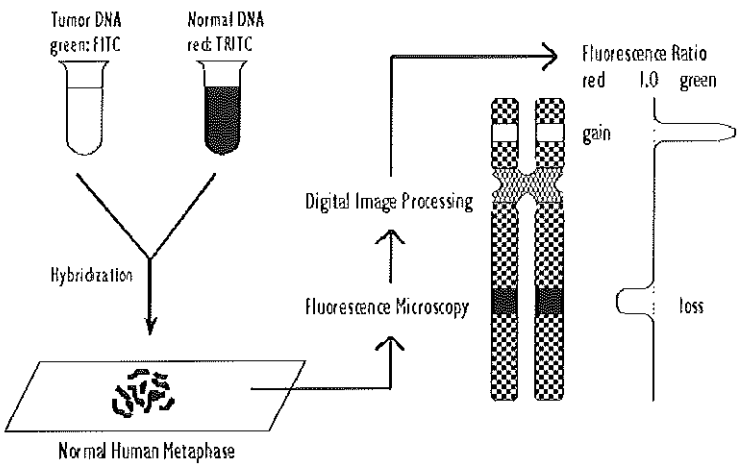

Fig.3: Schematic overview of the CGH technique. Tumor and reference DNA are labeled with a green and red fluorochrome, respectively, and hybridised to normal metaphase spreads. Images of the fluorescent signals are captured, and the green-to-red signal ratios are digitally quantified for each chromosome homologue. (reprinted with permission from Hermsen et al. 1996).

Another important breakthrough in the development and application of CGH is the use of archival (paraffin) material. In most studies test (tumor) DNA has been extracted from fresh or frozen samples and from cultured cell lines. Recently procedures were improved to isolate good quality DNA from paraffin-embedded material (Speicher et al. 1993; Isola et al. 1994). CGH with DNA extracted from paraffin-embedded material allows retrospective analysis of many tumors that are pathologically well characterised and of patients with known clinical outcome. The main difficulty with the use of paraffin-embedded material is to obtain DNA fragments which are suitable for CGH after labeling by nicktranslation. The optimal size of labeled DNA for CGH is 500-2000 bp, the same size that is often obtained of DNA extracted from paraffin-embedded material, or DOP (degenerated-oligo-primed)-PCR products. Nicktranslation will further reduce the fragment size by the use of DNase. To avoid this problem, a chemical labeling with platin can be used (Alers et al. 1999). These technical developments have made CGH more easily applicable for the analysis of solid tumors both from frozen and paraffin embedded material (see for review http://www.nhgri.nih.gov/DIR/LCG/CGH/index.html).

Depending on the type of aberration, the ploidy of the tumor cells, intra-tumor heterogeneity and contamination with normal cells of the specimens under investigation, CGH

allows identification of chromosomal gains and losses with a fair resolution. Amplifications are detectable at a higher resolution than deletions. Based on simulation models, an amplified region can be detected if the product of the size of the amplified region and the number of copies is at least 2 Mb (Piper et al. 1995). In this way CGH can detect an amplified region as small as 100 kb as long as the copy number is at least 20 (Joos et al. 1993). The detection limit of a deletion is in the order of 3 Mb using special detection criteria (standard reference intervals) (Kirchhoff et al. 1999). Since the main limitation of CGH is the resolution of the hybridisation target (the chromosomes) (see Table 1) , CGH has recently been applied on DNA and cDNA clones from small regions on the genome, arrayed on a slide, in so called micro-arrays (Pinkel et al. 1998; Pollack et al. 1999). This modification highly improves the resolution of the approach and can be considered as a bridge between cytogenetics and molecular genetics.

1.3 Testicular germ cell tumors; origin and development

Human germ cell tumors are a heterogeneous group of tumors of which the (primordial) germ cell is the cell of origin. They occur mainly in the gonads, but also in specific extragonadal sites. This distribution is explained by the migration route of the primordial germ cells, originating in the yolk sac endoderm of the 4th week embryo and migrating along the dorsal mesentery to the genital ridge. In the male, germ cell tumors may develop either in the testis or in midline sites of the body such as the sacrococcygeal region, retroperitoneum, mediastinum and brain (Mostofi 1973; Scully 1979). In adolescents and adults (including elderly) the most frequent site of is the testis. In neonates and infants the majority is found in the sacral region, predominantly affecting females. The testicular germ cell tumors can, based on their histology, age of the patient at clinical diagnosis and biological behaviour, be classified into three groups;

- Testicular germ cell tumors of neonates and infants (GCTI): teratomas and yolk sac tumors;
- Testicular germ cell tumors of adolescents and young adults (TGCT): seminomas and nonseminomas;
- Testicular germ cell tumors of the elderly man (SS): spermatocytic seminomas.

In the next paragraphs the main characteristics of these groups and the current view on their relationship(s) will be reviewed.

Testicular germ cell tumors of neonates and infants (GCTI)

GCTI are found in young children with an incidence of 0.12 per 100.000 (Looijenga 1999, for review). They occur mostly in extragonadal sites (67%), especially in the sacral region

(45%), and only occasionally in the testis (6%). Histologically, the GCTI are composed of teratoma or yolk sac tumor. The teratomas are benign and can be cured by orchidectomy alone. The yolk sac tumors, occurring more often than the teratomas (4:1) (Harms and Janig 1986), are malignant and may require additional chemotherapy.

In contrast to its presence in the adjacent parenchyma of TGCT (see below), carcinoma *in situ* (CIS), the common precursor of TGCT (Skakkebæk et al. 1987), has not been convincingly demonstrated adjacent to GCTI. Sacrococcygeal germ cell tumors are usually large, benign teratomas presenting at or shortly after birth. Patients older than 6 months of age have a greater risk of developing a malignant yolk sac tumor either within a pre-existing teratoma or at the site of a previously incompletely resected teratoma (Perlman et al. 1994).

Flow cytometry studies have shown that teratomas are in general diploid (Hoffner et al. 1994; Silver et al. 1994; Stock et al. 1995) while yolk sac tumors may be aneuploid, mainly near-tetraploid (Oosterhuis et al. 1989; Kommoss et al. 1990; Perlman et al. 1994; Jenderny et al. 1995). This ploidy difference was confirmed by cytogenetics: both FISH and karyotypic analyses showed no chromosomal aberrations in the teratomas and several aberrations in the yolk sac tumors. Whether this absence of chromosomal aberrations in the teratomas is due to loss of tumor cells upon handling is unknown thusfar. In the yolk sac tumors, karyotyping revealed anomalies of the short arm of chromosome 1, in particular loss of band p36, the long arm of chromosome 6 and abnormalities of 3p (Kaplan et al. 1979; Oosterhuis et al. 1988; Oosterhuis et al. 1993; Hoffner et al. 1994; Perlman et al. 1994; Bussey et al. 1999). FISH confirmed the chromosome 1 aberrations but demonstrated also copy number changes for chromosomes 8, 10, 12, 17 and X in the yolk sac tumors (Hu 1992; Stock et al. 1994; Jenderny et al. 1995; Perlman et al. 1996). No FISH data on chromosome 3 and 6 are available.

Testicular germ cell tumors of adolescents and adults (TGCT)

Epidemiology

Although TGCT are one of the rarer types of malignancies, accounting for only 1-3 % of all neoplasms in men, it is the most common cancer in young adult males (15 – 45 yrs) (Swerdlow 1993). The worldwide incidence has more than doubled over the past 40 years (Bosl and Motzer 1997). Several epidemiological studies show geographical and racial differences in the occurrence of these tumors. In European populations the incidence is about 5/100.000 while the incidence is lower in African and Asian populations (Parkin and Muir 1992) with exception of the New Zealand Maori, in whom the incidence is one of the highest in the world (8.3/100.000). There is also a wide variation within geographical regions: for instance, in 1980, Denmark showed a ten fold higher incidence than Lithuania (Adami et al. 1994). There is no explanation for these racial and geographic differences thus far. Interesting is the finding that cryptorchidism, which is a major risk factor (4-5x) (Pottern et al. 1985; Giwercman et al. 1987)

for the development of TGCT, is three times lower in black than in white males (Henderson et al. 1988). Other factors can be the onset of puberty, which occurs late in Maori boys, and genetic predisposition (Wilkinson et al. 1992). Suggestions that environmental factors are of etiological importance came from a study in Denmark. It showed that men born during the second world war have a lower risk for TGCT than those in birth cohorts preceding and following the war (Møller 1989). Recent publications suggest that the increasing presence of estrogenic compounds in the environment plays a role in the increasing incidence of TGCT (Sharpe and Skakkebaek 1993; Toppari et al. 1996).

Histology, clinical behaviour and models of progression

Clinically and morphologically, TGCT can be divided into two different entities: seminoma (SE) and nonseminoma (NS). SE becomes clinically manifest at a mean age of 35 years, while NS usually presents approximately one decade earlier. SE is composed of the neoplastic counterparts of early germ cells, probably primordial germ cells. NS may be composed of embryonic tissues (embryonal carcinoma, immature and mature teratoma) and/or extra-embryonic tissues (yolk sac tumor and choriocarcinoma). Of the TGCT, 40 % and 50 % present as NS and SE, respectively. The remaining TGCT, composed of both a SE and a NS component, are classified as combined tumor (CT) according to the British classification (Pugh 1976) and as NS according to the WHO classification (Mostofi et al. 1998).

In general, SE is less aggressive than NS, although the aggressiveness of the latter depends on the histologic subtype. The overall cure rate of patients with TGCT is high, but still about 10 % of the patients die of their disease. Aggressive treatment may lower this percentage. Thus far there are no reliable factors predicting outcome of therapy on an individual basis (Bokemeyer and Schmoll 1995).

SE and NS have a common precursor, known as CIS (Skakkebæk et al. 1987), composed of intratubular tumor cells resembling SE cells. Extrapolation, based on follow up studies of men diagnosed for CIS without an invasive TGCT, showed that CIS will always progress into an invasive TGCT (Burke and Mostofi 1988; Dieckmann and Loy 1993; Giwercman et al. 1993). The initial expansion of the CIS cells can be seen either as intratubular or micro-invasive seminoma.

Basically two models exist for the development of CIS into an invasive TGCT. One assumes that all histological variants of TGCT originate independently (Mostofi 1984; Sesterhenn 1985) while the other supposes SE to be an intermediate stage between CIS and the various nonseminomatous lineages (Oliver 1987; Oosterhuis et al. 1989). Cytogenetic studies favor the latter model with the finding of common structural chromosomal aberrations in both the SE and NS component of a CT (Haddad et al. 1988; Van Echten-Arends et al. 1995a). This model was recently adjusted with the hypothesis that in every stage during the development of TGCT, reprogramming of CIS or seminoma cells to pluripotent embryonal carcinoma cells, the stem cells of nonseminomas, can take place (Oosterhuis et al. 1997). It is suggested that the

chance of reprogramming of CIS and SE to a NS decreases with age, explaining the observation that the nonseminomas are clinically manifest at an earlier age than the seminomas (Oosterhuis et al. 1989).

Chromosomal constitution

Flow cytometric studies showed that TGCT are aneuploid tumors with SE being hypertriploid and NS hypotriploid (Oosterhuis et al. 1989; Fosså et al. 1991; El-Naggar et al. 1992). In contrast to the model proposed by Nowell (Nowell 1976; Nowell 1986) in which polyploidization is related to tumor progression, in TGCT polyploidization is most likely one of the first events, followed by net loss of chromosomal material. The propensity for polyploidization in TGCT may be related to the (primordial) germ cell derivation of these tumors. These cells may be particularly prone to polyploidization because they combine high mitotic activity and bridge formation. Interestingly, a defective mechanism of bridge formation has been implicated in the pathogenesis of TGCT (Gondos 1993).

Polyploidization and subsequent net loss of chromosomes in TGCT results in a specific pattern of copy numbers of chromosomes as found by karyotyping (De Jong et al. 1990; Castedo et al. 1989; Van Echten-Arends et al. 1995b). These studies showed, compared to a triploid DNA index, overrepresentation of chromosomes 7, 8, 12 and X and underrepresentation of chromosomes 11, 13, 18 and Y, both in SE and NS. Other frequently found chromosomal abnormalities are loss of chromosome 4, 5 and 9, gain of chromosome 21 and at a lower frequency (< 20%) deletions or derivatives of 1p21-26, 6q14-25, 7q11-q36 and 12q12-24 (Murty and Chaganti 1998). These are, however, not consistently found and occur also in other tumors, suggesting a tumor progression related event. Despite common aberrations found in SE and NS, a significantly higher copy number of chromosomes 7, 15, 19 and 22, and a significantly lower number of chromosome 17 was found in SE compared with NS (Van Echten-Arends et al. 1995b).

Besides the fact that TGCT are virtually always near-triploid, the only other consistent genetic anomaly is overrepresentation of the short arm of chromosome 12, mostly as an isochromosome of 12p [i(12p)] (see Figure 4). This isochromosome was first described in 1982 (Atkin and Baker 1982) and is found in about 80% of TGCT (De Jong et al. 1990; Sandberg et al. 1996). The uniparental origin of both chromosomal arms of i(12p) (Sinke et al. 1993) supports the idea that i(12p) originates from misdivision of the centromere rather than from a non-sister chromatid exchange as supposed by others (Mukherjee et al. 1991). Even though 12p aberrations are always present in TGCT, polyploidization precedes i(12p) formation. This was shown by the finding of retention of heterozygosity of the long arm of chromosome 12 in i(12p) positive TGCT (Geurts van Kessel et al. 1989).

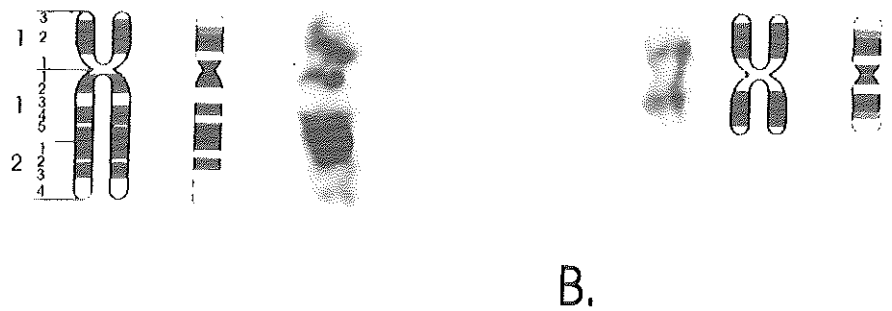

Fig.4: *Representatives of schematic and actual G-banding of a normal chromosome 12 (A) and an isochromosome 12p [i(12p)] (B).*

The idea that overrepresentation of the short arm of chromosome 12 is of importance in the development of TGCT was confirmed by FISH (Samaniego et al. 1990; Suijkerbuijk et al. 1993). It was shown that also the i(12p) negative TGCT show gain of 12p. Moreover, amplification of a restricted region of the short arm of chromosome 12 was found in a metastasis of a SE (Suijkerbuijk et al. 1994).

(Proto-)oncogenes and suppressor genes

Although information becomes available about the cytogenetic changes in the development of TGCT, molecular research in TGCT is still in its initial stage. To obtain more information about the possible role of tumor suppressor genes, loss of heterozygosity was addressed in several studies. Although contradictory results were obtained, some results were in accordance with cytogenetic data, e.g. deletions of regions involving 1p (Mathew et al. 1994), 5q (Murty et al. 1994a; Peng et al. 1995; Peng et al. 1999) and 12q (Murty et al. 1992).

In addition, some hotspots for deletions have been found, including *DCC* (18q21) and *RB1* (13q14) (Murty et al. 1994b). However, most tumor suppressor genes and (proto-)oncogenes show no or only a low percentage of aberrations in TGCT: e.g. *MYC* (*c-MYC*; 8q24, *N-MYC*; 2p24), *c-KIT* (4q11-12) (Tian et al. 1999) and *P53* (17p13) (Lothe et al. 1995), *K-RAS* (12p12) (Dmitrovsky et al. 1990) and *NME1* (17q22) (Schmidt et al. 1997).

At the RNA level, increased expression has been found of the proto-oncogenes *c-KIT* (Rajpert-De Meyts and Skakkebæk 1994; Strohmeyer et al. 1995), N-*MYC*, c-*MOS* (Shuin et al. 1994), cyclin D2 and K- or N-*RAS* (Houldsworth et al. 1997). A decrease or loss of expression was found for *RB*, *DCC* (Murty et al. 1994b), *INT-2* (Yoshida et al. 1988; Shimogaki et al. 1993) and *c-ERB-1* and *2* (Shuin et al. 1994).

Since no microsatellite instability has been found thusfar with one exception (Murty et al. 1994c), there is no indication that defects of repair genes participate in the development of TGCT (Lothe et al. 1995).

Familial TGCT

Epidemiological and linkage analyses suggest that one third of all TGCT-patients may be genetically predisposed (Nicholson and Harland 1995). Several investigators have tried to identify the chromosomal regions harboring the susceptibility gene(s). Linkage analysis of 50 families with 221 markers, (Leahy et al. 1995) identified regions on chromosomes 1, 4 (2 regions), 5, 14 and 18 as candidates. Re-analysis of this series and combining the results with those of an extended study by the International Testicular Cancer Linkage Consortium, provides suggestive evidence for linkage to parts of chromosomes 3, 5, 12 and 18 (Rapley et al. 1998). Unfortunately, the results do not provide strong evidence for the location of a predisposing gene, for which genetic heterogeneity (different genes causing the same disease endpoint) might be an explanation. A recent study indicated linkage to Xq27 in case of bilateral TGCT and TGCT related to cryptorchidism (Rapley et al., 2000).

Germ cell tumors of the elderly man (SS)

The third type of germ cell tumor which can be distinguished in the testis is spermatocytic seminoma (SS), predominantly found in elderly men, usually over 50 years of age. They never arise in extra-testicular sites, and mostly run a benign course (Talerman 1980; Burke and Mostofi 1993). Phenotypically, SS is characterised by cellular heterogeneity with large, intermediate and small cells. Morphologically they can mimic SE (Talerman 1980). In contrast to the SE, SS are supposedly derived from more differentiated cells: the spermatogonia B (Masson 1946; Rosai et al. 1969; Romanenko and Persidskii 1983). CIS can not be found in the adjacent parenchyma of these tumors, although intratubular SS, supposedly the pre-invasive precursor of this neoplasm, is often present (Muller et al. 1987).

Although in general a separate pathogenesis for SS is accepted, there are still some doubts. Based on the finding of c-KIT positivity in 40% of SS, it was most recently suggested that at least some of the SS originate more directly from primordial germ cells (Kraggerud et al. 1999). Immunohistochemical detection of PLAP indicated clear differences between the different histologies. SS were negative (Dekker et al. 1992; Burke and Mostofi 1993) while CIS and SE were positive (Manivel et al. 1987). Flow cytometry showed a heterogeneous pattern for SS including both diploid and aneuploid stem lines (Takahashi 1993, Looijenga et al. 1994). Because of their rarity (0.2 per 100.000) (Burke and Mostofi 1993) little is known about their chromosomal constitution.

1.4 Aims of this thesis

This thesis aims at a further understanding of the molecular genetics of germ cell tumors of the testis. In particular, the three entities (GCTI, TGCT and SS) are investigated regarding their chromosomal constitution using karyotyping, CGH and FISH. The results will be used to identify chromosomal abnormalities involved in the development of these types of cancer, as a first step in the identification of the involved genes. In addition, the results will be used to discuss the possible pathogenetic relationship between these tumor types.

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Chapter 2

Comparative genomic and *in situ* hybridization of germ cell tumors of the infantile testis

Submitted

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Abstract

Chromosomal information on germ cell tumors of the infantile testis, i.e. teratomas and yolk sac tumors, is limited and controversial. We studied two teratomas and four yolk sac tumors using comparative genomic- and *in situ* hybridisation (CGH and FISH). No chromosomal anomalies were found in the teratomas by any of the methods, not even after CGH on microdissected tumor cells. All yolk sac tumors showed aneuploidy, loss of parts of 4q and 6q, and gain of parts of 20q. Underrepresentation of parts of 8q, and overrepresentation of parts of 3p, 9q, 12p, 17, 19q, and 22 were detected in most cases. In addition, one recurrent yolk sac tumor after a sacral teratoma was studied, showing a highly similar pattern of imbalances. While CGH demonstrated loss of 1p36 in one testicular yolk sac tumor, FISH revealed loss of this region in all yolk sac tumors. High level amplification of the 12q13-q14 region was found in one yolk sac tumor. MDM2, of which the encoding gene maps to this chromosomal region, was found in all cases using immunohistochemistry, while no P53 could be detected. Accordingly, no mutations within exons 5-8 of the *P53* gene were observed.

These data prove absence of gross chromosomal aberrations in teratomas of the infantile testis, and show a characteristic pattern of gains and losses in the yolk sac tumors. Besides confirmation of previously found anomalies, the recurrent losses of 1p21-31, 4q23-33 and gains of 9q34 and 12p12-13 are novel. While genetic inactivation of P53 seems unimportant in the pathogenesis of these tumors, biochemical inactivation by MDM2 might be involved. These data support the existence of three entities of germ cell tumors of the human testis, i.e. teratomas and yolk sac tumors of infants, seminomas and nonseminomas of adolescents and young adults, and spermatocytic seminomas of elderly, each with their own specific pathogenesis.

Introduction

Human germ cell tumors comprise a heterogeneous group of neoplasms, which can be found at different anatomical localizations (gonads and several extragonadal sites), and present clinically at different ages (Oosterhuis et al., 1997; Looijenga and Oosterhuis 1999, for review). Within the testis, three entities can be distinguished. The most common types are those of adolescents and young adults: the seminomas and nonseminomas (Mostofi et al., 1987). They originate from carcinoma *in situ* (CIS) (Skakkebæk 1972), which can be frequently found in preserved parenchyma adjacent to an invasive tumor. Both seminomas and nonseminomas are characterized by consistent gain of the complete short arm of chromosome 12 (Sandberg et al., 1996, for review). In addition, these tumors express wild type P53 extensively (Schenkman et al., 1995; Guillou et al., 1996). Less common are spermatocytic seminomas, mostly found in elderly men (Burke and Mostofi 1993). We demonstrated recently using karyotyping and comparative genomic hybridisation (CGH) that these tumors are characterised by gain of chromosome 9 (Rosenberg et al., 1998). The third group of testicular germ cell tumors comprise those found at young age, histologically divided into teratoma and yolk sac tumor, although other histological elements have sporadically been reported.

Information about the chromosomal constitution of germ cell tumors of infants, including those of the testis, is limited, mainly due to their rareness, and sometimes inconsistent. Flow cytometry demonstrated in general that teratomas are diploid, whereas yolk sac tumors can either be diploid, tetraploid or aneuploid (Kommoss et al., 1990; Silver et al., 1994). This difference was confirmed by karyotyping, showing no aberrations in teratomas, while anomalies of the short arm of chromosome 1, in particular loss of band p36, the long arm of chromosome 6, and abnormalities of 3p were observed in yolk sac tumors (Kaplan et al., 1979; Oosterhuis et al., 1988; Oosterhuis et al., 1993; Hoffner et al., 1994; Perlman et al., 1994; Stock et al., 1995; Stock et al., 1996; Bussey et al., 1999). *In situ* hybridisation supported absence of numerical abnormalities in teratomas, but demonstrated in addition to chromosome 1 aberrations, also copy number changes for chromosomes 8, 10, 12, 17, and X in the yolk sac tumors (Hu et al., 1992; Stock et al., 1994; Jenderny et al., 1995; Perlman et al., 1996). The cytogenetic studies so far include only a few informative cases, possibly explained by loss of tumor cells during sample-preparation. Even the largest study on pediatric germ cell tumors (Bussey et al, 1999) reported only one testicular yolk sac tumor, present in a patient under the age of 10 years, with an abnormal karyotype. Furthermore, conflicting data with respect to 12p (Perlman et al., 1994; Stock et al., 1995; Jenderny et al., 1995) and the presence of CIS (Stamp and Jacobsen 1993; Jørgensen et al., 1995; Hawkins et al., 1997) have been found. These uncertainties fluel the discussion about the pathogenetic relationship between germ cell tumors of the infantile testis and seminomas and nonseminomas.

We performed fluorescent *in situ* hybridisation (FISH) and CGH on a series of two teratomas and four yolk sac tumors of the infantile testis. Both teratomas were also karyotyped. In addition, a recurrent yolk sac tumor of a sacral teratoma was studied. CGH can be performed

on fresh, frozen and formalin-fixed (archival) material, and allows a screen of chromosomal imbalances throughout the whole genome within a single experiment (Kallioniemi et al., 1992). This method was previously found to be highly informative in our hands, even when applied on a relative small number of cases (Rosenberg et al., 1998). In case of absence of imbalances, CGH was also done with DNA from microdissected tumor cells. The role of P53 and MDM2 was investigated by immunohistochemistry and mutation analysis of exons 5-8 of *p53*.

Material and methods

Samples

The samples included in this study were collected in close collaboration with urologists and pathologists in the south-western part of the Netherlands and the University Hospital in Groningen. Directly after surgical removal, representative parts of the tumor and adjacent normal tissue (when available) were snap frozen and other pieces were fixed overnight in 10% buffered formalin and subsequently embedded in paraffin. Of one case (no. 4) only paraffin-embedded material was available. The tumors were diagnosed according to the World Health Organisation classification for testicular tumors (Mostofi 1980). Two cases were pure mature teratomas, while four were pure yolk sac tumors. In addition, we studied one recurrent yolk sac tumor of a sacral teratoma which we reported previously (Oosterhuis et al., 1993).

Metaphase preparations

Representative parts of the tumor were enzymatically digested (collagenase, Sigma), and cultured in T75 flasks (Costar) for several days under standard conditions, i.e. 37°C in a humidified atmosphere of 5% CO_2 in DMEM/HF12 with 10% heat-inactivated FCS (BRL-GIBCO), as reported before (Castedo et al, 1989). Mitotic cells were harvested after 2-4 hours of colcemid treatment, swollen in hypotonic solution, and fixed in methanol:acetic acid (3:1). For conventional G-band karyotyping, air-dried preparations were digested with pancreatin (Sigma). The chromosome constitution was described according to the International System for Human Cytogenetic Nomenclature 1995 (Mitelman 1995).

For CGH, metaphase spreads were prepared using standard procedures from *in vitro* phytohemagglutinin-stimulated peripheral blood lymphocyte cultures of a healthy male.

Comparative Genomic Hybridisation

High molecular weight DNA was isolated from the snap frozen tissue samples (test DNA) and from peripheral blood of a normal male (reference DNA) using standard procedures (Maniatis et al., 1982). DNA of the paraffin-embedded yolk sac tumor was isolated as described before (Alers et al., 1997). In case no aberrations were identified on the initial DNA sample (both teratomas), DNA of purified tumor cells was acquired from frozen tissue as follows. Tissue sections of 20 micron thickness of the samples were cut and air dried. In addition, a hematoxylin and eosin staining was done of a parallel tissue section of 4 micron thickness for additional histological control. The cells of interest were identified using an inverted microscope (Axiovert 10, Carl Zeiss) and microdissected with a self-made glass capillary piece. The section after microdissection was also stained for histological check. The tissue parts were directly put into a reaction vessel, and dissolved in TNE (10 mM Tris, 400 mM NaCl, 2 mM EDTA pH 8,2). Proteinase K (10 mg/ml) and SDS (10%) was added, and incubation was done overnight at 37C. Subsequently, DNA was extracted with phenol/chloroform/ isoamylalcohol

(25:24:1) (Merck) and precipitation was performed with glycogen (10 mg/ml) (Boehringer) as carrier. Pellets were dissolved in TE (Tris 10 mM, EDTA 0,1 mM, pH 8,0). Eight to 25 ng of DNA from each microdissected teratoma sample and from a normal male were amplified by standard DOP-PCR (Telenius et al., 1992). The quality of this approach was checked with control DNA amplified with the same procedure derived from normal males (XY) and females (XX). In total seven independent experiments were performed, and all showed a balanced profile for the autosomes. The sex chromosomes showed the expected imbalances in gender-mismatched hybridisations. In addition, CGH was performed on an osteosarcoma of a patient with Rothmund-Thomson syndrom, both on genomic DNA without, as well as after DOP-PCR. From the 20 imbalances found in the genomic DNA, 18 were observed after amplification (two false negatives). No aberrations were detected only in the sample after amplification (no false postives). This method was reproducibly applicable on 8 ng DNA, equivalent to about 900 nuclei with a diploid DNA content (not shown). Of the samples studied, DNA equivalent of at least 1500 nuclei was used for the amplification. This approach was successfully used to investigate the chromosomal imbalances of various developmental stages of both seminoma and nonseminoma, including CIS (Looijenga et al, 2000).

The CGH procedure and analysis was performed as described before (Rosenberg et al., 1998). Both the control male DNA and the tumor DNA were directly labelled by nick-translation with lissamine and FITC respectively. The data were analysed using Quips XL software from Vysis (Downers Grove, IL). Normalisation was carried out using the average of the green to red fluorescent intensities for the entire metaphase. Five metaphases were studied for each case. Losses of DNA sequences were defined as chromosomal regions where the average green-to-red ratio and its 95% CI are below 0.9 while gains are above 1.1. These narrow thresholds of 0.9 and 1.1 are suitable for the direct labelling procedure, as used in this study (see above), as discussed by us before (Rosenberg et al., 1998). The results obtained, with and without DOP-amplification, resulted in narrow 95% confidence intervals (not shown and Looijenga et al., 2000). The heterochromatic blocks of chromosome 1, 9, 16 and Y, and the immediate telomeric regions were excluded from the analysis because these regions present variable results in experiments with normal control DNAs.

***In situ* hybridisation**

FISH was performed with a chromosome 13 probe (YAC 908C2, mapped to 13q34) (Kingsley et al., 1997) kindly provided by B. Eussen (Department of Clinical Genetics, Erasmus University Rotterdam). Since chromosome 13 did not show imbalances in any of the tumors by CGH, combination of the FISH and CGH data made discrimination between diploid and polyploidy possible. This approach has been found to be informative in previous studies on a cell line (Rosenberg et al., 1998) and invasive seminomas and nonseminomas (Rosenberg et al. in press).

Chromosome 1 band p36 is a critical region for CGH. Therefore possible loss of the 1p36

region was studied in addition to CGH in more detail by means of a double FISH approach using a probe specific for the centromeric region of chromosome 1 (Cook and Hindley 1979), as well as a probe specific for the 1p36 region (cosmid: D1S32, pBG2.8, kindly provided by Prof. dr.A. de Paepe, Centre of Medical Genetics, University of Gent, Belgium). Representative formalin-fixed paraffin-embedded tissue section of 5 micron thickness were used. The procedure used is a slightly modified method described previously (Hopman et al., 1992). Briefly, the sections were deparaffinized by xylene and methanol, pretreated in 1M NaSCN at 80°C and pepsin (8000U/ml) at 37°C respectively followed by rinsing in distilled water and PBS and dehydrated. Probes were labelled by nick-translation, according to standard procedures, either with dioxigenin-11-dUTP (Boehringer Mannheim, Germany) or biotin-16-dUTP, and applied in 10-15 ul hybridisation mixture on the tissue slides (13q probe: 20 ng/ul, 1p36: 2 ng/ul and chromosome 1 centromere 2 ng/ul). The probes were denaturated together with the target by placing the slide for 10 min. on the bottom of a 80°C oven. After hybridisation overnight at 37°C, the slides were washed stringently and the hybrids were detected by FITC-conjugated sheep-anti-digoxigenin (Boehringer Mannheim, Germany) and CY3-conjugated avidine (Jackson Immuno Research laboratories, Inc. Westgrove, PA, USA) as described by us before (Mostert et al., 1998). Results were studied with a Zeiss Axioskop fluorescence microscope (Carl Zeiss Jena, Jena, Germany) with a Pinkel filter in combination with a triple band-pass filter to visualise FITC, CY3 and DAPI simultanously. This set of probes was verified to be usefull on a series of normal tissues, showing a 1:1 ratio (not shown), as well as on host cells within the same histological section of which the tumor cells were investigated. The tumor component was only scored when a 1:1 ratio was found in the controls. In total 30 nuclei of each component were investigated, of which possible differences were investigated using Student' t test analysis.

***P53* mutation analysis**

Since the majority (78%) of the missense mutations in the *p53* gene are found in exons 5-8, the tumors were screened for mutations in this region using PCR amplification and subsequent single strand conformation polymorphism analysis (SSCP) as described previously (Schuyer et al., 1998). Briefly, the specific exon regions were amplified using commercially available primers (Clontech, Palo Alto, CA, USA) and analysed by SSCP. PCR products showing an altered electrophoretic mobility were re-amplified and sequenced by double stranded sequencing.

Immunohistochemistry

Immunohistochemistry was performed on representative formalin-fixed, paraffin embedded tissue section of 3 micron thickness of both teratomas and all yolk sac tumors. The P53 specific antibodies (DO-7, DAKO A/S, Denmark) were used in a final concentration of 80 ug/ml and the MDM2 specific antibodies (clone SMP14, NeoMarkers, Union City, USA) in a final concentration of 40 ug/ml. The sections were pretreated with a modified heat induced

antigen retrieval method (Shi et al., 1991) using 10 mmol/l citrate buffer pH 6.0. The detection was done with the avidin-biotin-peroxidase complex (DAKO A/S Denmark) according to the manufacturer's instructions using 3,3'-diaminobenzidine as chromogen. Sections were lightly counterstained with hematoxyline. In all experiments, both positive (samples with known presence of P53 and MDM2) and negative controls (samples without the presence of P53 and MDM2, and without the first antibody) were included. Photographs were made using an Olympus BX 50 microscope, equiped with a Olympus DP 10 camera and the Olympus DP-Soft analysis system version 3.0 for Windows (Germany).

Results

The age of the patients at clinical presentation was 10 months for both teratomas, and 16, 18, 21, and 24 months for the yolk sac tumors. Both teratomas presented at stage I, and were treated with surgery only. The patients were disease free 80 and 84 months after initial diagnosis. All testicular yolk sac tumors presented also at stage I. After surgical removal of the affected testis, the elevated alpha-fetoprotein level decreased according to half-life. Within several months, however, a gradually elevated alpha-fetoprotein level was observed in two patients (cases no. 4 and 5), and multiple metastases in lung, abdomen, and lymph nodes adjacent to the left kidney and the para-aortic region were identified. Chemotherapeutic treatment resulted in complete remission both by marker analysis and scanning techniques, although follow-up is less than 1.5 years. The other two patients (no. 1 and 2) are still disease free 6 and 12 years after initial diagnosis. Testicular parenchyma could be observed in all cases, in which immature germ cells, but no CIS, were found (not shown). In addition, one recurrent yolk sac tumor of a sacral teratoma (case no. 3) was investigated. The teratoma was diagnosed at birth, whereas the yolk sac tumor presented 19 months later with multiple metastases in lymph nodes in pelvic and spinal cord region. After chemotherapy, a residual mature teratoma was found after surgical intervention. Twelve years after initial diagnosis, the patient is still disease free.

Classical karyotyping after short term *in vitro* culturing showed 46,XY for both testicular teratomas. The sacral teratoma was previously found to be diploid, aneuploidy was observed in the recurrent yolk sac tumor (Oosterhuis et al., 1993). None of the testicular yolk sac tumors were karyotyped successfully. No chromosomal aberrations were detected with CGH in the teratomas using DNA isolated from the total sample. Therefore the tumor cells were specifically purified from frozen tissue sections using microdissection (see Figure 1), of which DNA was isolated and used again for CGH (see **MATERIAL AND METHODS** section).

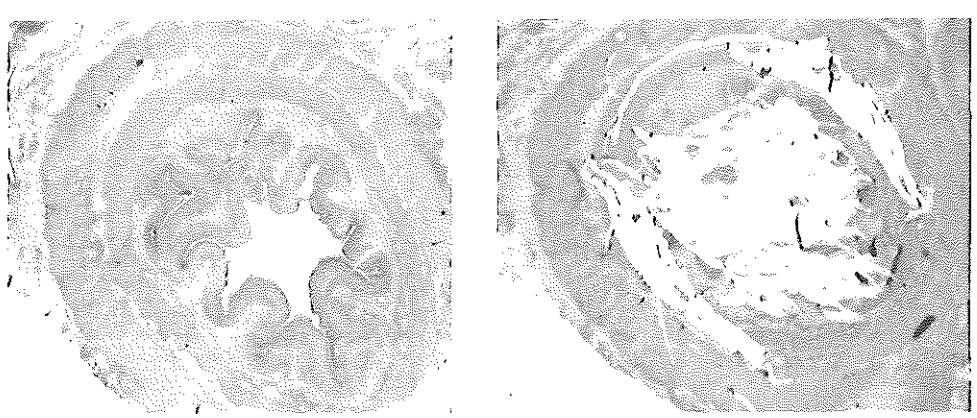

Fig. 1: Representative example of a frozen tissue section of a teratoma of the infantile testis, before (left panel) and after (right panel) microdissection.

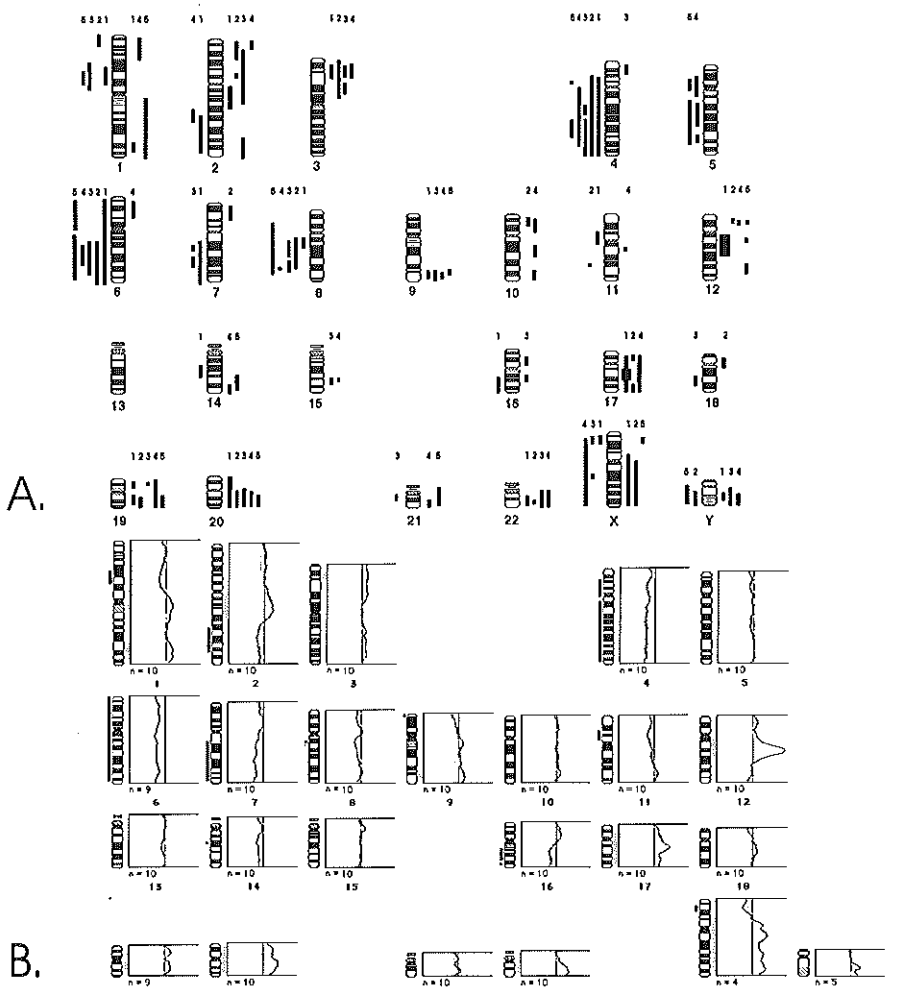

Fig. 2A: Summary of the chromosomal imbalances detected by CGH in four yolk sac tumors of the infantile testis (no. 1, 2, 4, and 5) and one recurrent yolk sac tumor of a sacral teratoma (no. 3). DNA of all cases, except case no.4, was isolated from snap frozen tissue. DNA of case no. 4 was obtained from formalin-fixed, paraffin-embedded tissue. CGH was performed on DNA isolated directly from the samples, without amplification. Lines on the left and right side of the ideograms indicate under- and overrepresentation of chromosome material. The thick lines indicated the regions of high level amplification. The numbers on top of each line refer to the case analysed. B: average profiles of case no. 1 (see also Figure 2A). Gains of part of 3p, 9q, 17, 19q, 20q, and 22, and losses of parts of 4 and 6, as well as the amplification of a discrete region on the long arm of chromosome 12 and 17 can be seen.

Even using DNA isolated from these enriched tumor cells no chromosomal imbalances were observed (not shown). As indicated previously (Rosenberg et al., 1998 and Rosenberg et al, 2000) FISH with a specific probe against a chromosome showing a balanced pattern in CGH is informative to obtain more information about the ploidy of the cells under investigation. FISH using a chromosome 13-specific probe revealed only tumor cells with two or less signals per interphase nucleus (mean 1.8/SD 0.46 and 1.9/0.58, respectively, not shown), supporting diploidy. Because of the high percentage of tumor cells in the yolk sac tumors (more than 80%, see Figure 4 right panel for a representative example), no microdissection of the tumor cells and subsequent DNA amplification prior to CGH has been performed for these samples. All cases showed chromosomal imbalances, summarized in Figure 2A, of which corresponding profiles are shown in Figure 2B. Except for chromosome 13, all chromosomes showed imbalances at least in one of the samples. In particular, loss of parts of 4q and 6q, and gain of parts of 20q were observed in all cases, and gain of parts of 3p, 9q, 12p (see below), 17, 19 and 22, and loss of parts of 8q was observed in the majority of them. The yolk sac tumors which developed distant metastases (cases no. 4 and 5) showed specifically loss of parts of chromosome 5 and gain of parts of 14q and chromosome 21. Although the recurrent yolk sac tumor after a sacral teratoma (case no. 3) showed a highly similar pattern of imbalances as found for the primary testicular yolk sac tumors (see Figure 2A), loss of a part of 18q and gain of a part of 4p was specifically observed in this tumor. Using FISH with the chromosome 13 specific probe, all yolk sac tumors (except case no. 2, of which no appropriate material was available anymore) demonstrated tumor cells with three or more signals (between 12 and 30%) for chromosome 13, supporting aneuploidy (not shown).

CGH showed loss of the 1p21-p31 region in two testicular yolk sac tumors and the recurrent case of a sacral teratoma, while loss of 1p36 was found in only one of the testis. Double FISH using a probe for the centromeric region of chromosome 1 in combination with a 1p36-specific probe showed significant loss of the 1p36 region in all yolk sac tumors, but not the teratomas (see Table 1 and Figure 3). This particular set of probes showed a 1:1 ratio in all controls included (not shown).

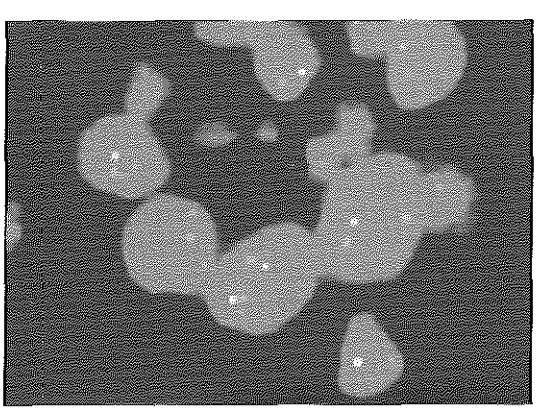

Fig. 3: Representative example of the double fluorescent in situ hybridisation with a probe specific for the centromeric region of chromosome 1 (red signal), and one for the 1p36 region (green signal) on a formalin-fixed, paraffin-embedded yolk sac tumor of the infantile testis (case no. 4). Note the reduced number of signals of the 1p36 region (green) compared to the centromere (red) (see also Table 1).

Table 1.

Results of the double fluorescent in situ hybridisation with a probe specific for the centromeric region of chromosome 1 and 1p36. The samples includes formalin-fixed, paraffin-embedded tissue of two teratomas, three yolk sac tumors of the infantile testis (case no. 2 was not studied), and one recurrent yolk sac tumor after a sacral teratoma (case no. 3). (Mean, standard deviation (between brackets), and corresponding p-values of Student's t analysis are indicated)

Histology:	Case:	Centromere:	1p36:	p-value
Teratoma:				
	A	1.7 (0.60)	1.9 (0.57)	NS[#]
	B	1.8 (0.44)	1.8 (0.38)	NS
Yolk sac tumor:				
	1	2.0 (0.64)	1.5 (0.82)	<0.001[*]
	3	1.5 (0.57)	0.9 (0.31)	<0.001[*]
	4	3.8 (0.90)	2.0 (0.29)	<0.001[*]
	5	3.1 (0.82)	2.1 (0.71)	<0.001[*]

[#]NS = not significantly different; [*]significantly different

One testicular yolk sac tumor (case no.1) showed high level amplification of the 12q13-q14 region, and although at a lower level, of region 17q12-q21 (see Figure 2B). Immunohistochemistry showed that all teratomas and yolk sac tumors are positive for MDM2 (see Figure 4), of which the corresponding gene maps to 12q13-q14. No p53 protein could be observed (see Figure 4), and no mutation within exons 5-8 of this gene were found (not shown).

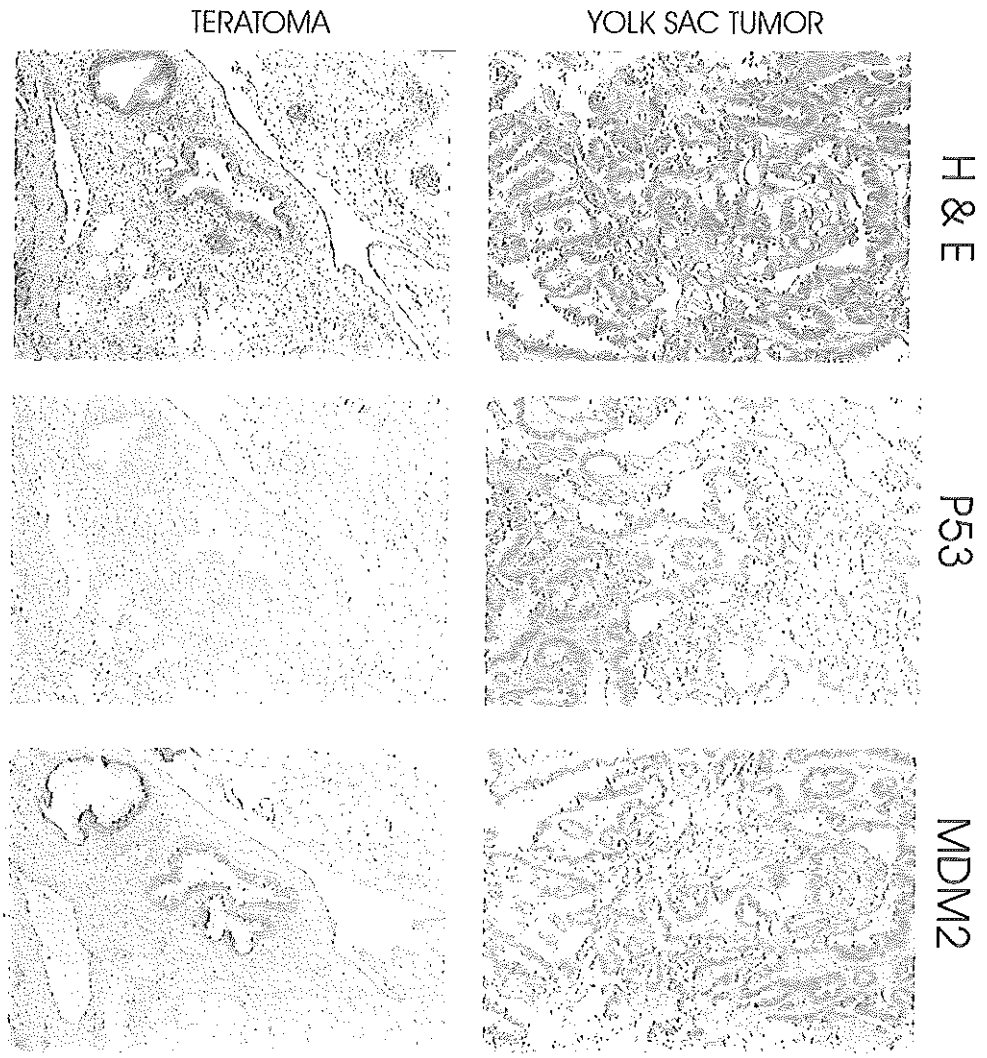

Fig. 4: *Representative examples of the immunohistochemical detection of the P53 protein (middle panel) and MDM2 protein (lower panel) on a tissue section of a formalin-fixed, paraffin-embedded teratoma (left panel) and yolk sac tumor (right panel) of the infantile testis. No P53 could be detected, while all tumor cells showed the presence of MDM2. The corresponding hematoxylin and eosin (H&E) stained slide is represented in the upper panel of the illustration. (magnification 100 X)*

Discussion

The pathogenetic relationship between the rare testicular germ cell tumors of infants and the more frequent testicular germ cell tumors of adolescents and young adults, i.e., seminomas and nonseminomas, is a matter of debate. This is partly because of misdiagnosis of testicular germ cell tumors of adolescents and young adults of especially young patients as those of the infantile testis. In our study, the oldest patient was 24 months of age at clinical diagnosis, thereby excluding this possibility. Indeed, the histologies found in this series were only teratoma and yolk sac tumor, as suggested before to occur in this particular subgroup (Oosterhuis et al., 1997). In addition, none of the cases showed CIS in the adjacent parenchyma, being the precursor cells of seminomas and nonseminomas (Skakkebæk 1972).

Information about the chromosomal constitution of germ cell tumors of the infantile testis is limited, and on some points contradictory. Both flow cytometry and karyotyping suggested that teratomas of the infantile testis are diploid without gross chromosomal aberrations (Kaplan et al., 1979; Silver et al., 1994; Stock et al., 1995). To exclude misinterpretation due to loss of tumor cells during sample preparation, we performed CGH and FISH. No chromosomal aberrations were observed, not even using DNA for CGH derived from microdissected tumor cells. These results prove for the first time that teratomas of the infantile testis are diploid without gross numerical chromosomal anomalies. In contrast, chromosomal aberrations were observed in the yolk sac tumors. In particular, loss of parts of 4q (23-33) and 6q (16-22), and gain of parts of 20q was found in all cases, and loss of parts of 1p (21-31) and 8q (23), and gain of parts of 3p (22-24), 9q (34), 12p (12-13), 17q (22-25), 19q (13), and 22 (13) was present in at least three (the minimal overlapping regions are indicated between brackets). These data provide a clear overview of chromosomal imbalances most often found in yolk sac tumors of the infantile testis. Several of these aberrations are in accordance to earlier findings. In particular, loss of 6q and 3p aberrations have been consistently found in the few karyotyped cases (Oosterhuis et al., 1988; Oosterhuis et al., 1993; Perlman et al., 1994; Bussey et al., 1999). In addition, imbalances of (parts of) chromosome 8, 17, 19, 20 and 22, have been reported occasionally (Oosterhuis et al., 1988; Jenderny et al., 1995). Loss of the 1p36 region, consistently found in infantile yolk sac tumors, including those of the testis (Perlman et al., 1994; Stock et al., 1995; Jenderny et al., 1995; Perlman et al., 1996; Bussey et al., 1999), is supported by our FISH data. However, the recurrent aberrations anomalies found on chromosomes 1p (21-31) and 4q (losses) and 9q and 12p (12-13) (gains) are novel. Noteworthy, development of distant metastases might be related to loss of parts of chromosome 5, and gain of parts of 14q and 21, which serves further investigation.

The recurrent yolk sac tumor of a sacral teratoma included in this study showed a highly similar pattern of imbalances compared to those of the infantile testis, in accordance to two cases studied by karyotyping (Perlman et al., 1994). This suggests that the chromosomal make up of this tumor is more associated with histology than with anatomical site. We recently performed CGH on a pure yolk sac tumor component of a mixed nonseminoma of the adult

testis (Looijenga et al, 2000). Although in general this yolk sac component showed a different pattern of chromosomal losses and gains compared to the results found in this study, loss of 6q was an exception. This anomaly was specifically found in the yolk sac tumor component of the nonseminoma of the adult testis, as well as in all yolk sac tumors included in this study, which suggests that it is related to yolk sac histology.

Three of the yolk sac tumors of the infantile testis showed overrepresentation of the 12p12-13 region specifically, which might explain the inconsistency found in literature (Perlman et al., 1994; Jenderny et al., 1995; Stock et al., 1995). Gain of the complete short arm of chromosome 12 is the most recurrent, and characteristic, chromosomal aberrations in testicular germ cell tumors of adolescents and young adults (Sandberg et al., 1996, for review). In addition, high level amplification of the 12p11.2-12.1 region can be found additionally (Mostert et al., 1998). Moreover, both seminomas and nonseminomas are consistently aneuploid (Oosterhuis et al., 1989; Van Echten-Arends et al., 1995; Mostert et al., 1996; Rosenberg et al., 1999), with a characteristic pattern of numerical aberrations: gain of parts of chromosomes 7, 8, 12 (short arm), and X, and loss of (parts of) 4, 5, 11, 13, 18, and Y. We also showed that gain of chromosome 9 is specifically found in spermatocytic seminomas (Rosenberg et al., 1998). These patterns are clearly different from that found in the yolk sac tumors (and teratomas) of the infantile testis as reported here.

The available data so far favour independent pathogenetic pathways for germ cell tumors of the infantile testis and those of the adult testis, i.e., seminomas, nonseminomas and spermatocytic seminomas. This is also supported by the absence of P53 both in teratomas and yolk sac tumors, whereas it has been consistently found in seminomas and nonseminomas (Schenkman et al., 1995; Guillou et al., 1996). Because of this finding, and absence of mutations within exons 5-8 of this gene, genetic inactivation of p53 in infantile testicular germ cell tumors is unlikely, although presence of the MDM2 protein might inactivate P53 by proteolytic breakdown (Prives 1998, for review). Amplification of the 12q13-q14 region, as found in one of the yolk sac tumors studied, and the immunohistochemical data, are compatible with such an involvement of *MDM2*.

In conclusion, the three entities of testicular germ cell tumors, i.e., teratomas and yolk sac tumors of infants, seminomas and nonseminomas of adolescents and young adults, and spermatocytic seminomas have separate pathogenetic pathways, associated with characteristic chromosomal anomalies. Application of FISH with probes for the chromosomal regions specifically lost or gained in the different groups of testicular germ cell tumors might be applicable in a diagnostic setting.

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Chapter 3

Chromosomal constitution of human spermatocytic seminomas

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Chromosomal Constitution of Human Spermatocytic Seminomas: Comparative Genomic Hybridization Supported by Conventional and Interphase Cytogenetics

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No data on the chromosomal constitution of spermatocytic seminomas are available thus far because of their rarity. Ploidy analysis performed on paraffin-embedded cases showed varying results from (near-) diploid to aneuploid. We applied comparative genomic hybridization on four snap-frozen primary spermatocytic seminomas of three different patients. Conventional cytogenetic analysis was successful in one, and "interphase cytogenetics" with centromeric region-specific probes was applied to another. The results from comparative genomic hybridization showed almost exclusively numerical chromosomal aberrations, in agreement with the data from karyotyping. Despite the limited number of cases studied, a nonrandom pattern of chromosome imbalances was detected: chromosome 9 was gained in all spermatocytic seminomas. This suggests that that this aberration plays a role in the development of this cancer. Interphase cytogenetics shows that the copy number of most chromosomes ranges from two to four, with an average of near trisomic. This constitutes the first report on the chromosomal constitution of spermatocytic seminomas. *Genes Chromosomes Cancer 23:286–291, 1998.*

INTRODUCTION

Spermatocytic seminomas are rare testicular tumors of germ cell origin that occur preferentially in elderly men (Burke and Mostofi, 1993; Cummings et al., 1994). In general, they are benign (Talerman, 1980; Talerman et al., 1984; Burke and Mostofi, 1993), and bilateral presentation has been reported (Talerman, 1980; Burke and Mostofi, 1993). Due to the low incidence of spermatocytic seminomas, cytogenetic analyses have not been reported on these tumors to date. The only chromosomal data available are the modal chromosome numbers of two cases that were described in 1973 (Atkin, 1973) which showed 52 and 82 chromosomes. However, ploidy analyses by means of flow cytometry and image analysis showed varying and sometimes conflicting results (Talerman et al., 1984; Dekker et al., 1992; Takahashi, 1993; Looijenga et al., 1994).

We applied comparative genomic hybridization (CGH; Kallioniemi et al., 1992) on four snap-frozen primary spermatocytic seminomas from three different patients. One of the spermatocytic seminomas was analyzed successfully also by using cytogenetic analysis without in vitro culture, and that case constitutes the first in the literature. Because no ploidy information can be associated with CGH results without complementary studies, in addition, we investigated one of the spermatocytic seminomas by using fluorescence in situ hybridization (FISH) with centromeric-specific probes.

MATERIALS AND METHODS

Samples

Testicular samples were collected in collaboration with Departments of Urology and Pathology at institutions in the Southwestern part of the Netherlands. After incision, representative samples were snap frozen, formalin fixed, and, subsequently, paraffin embedded. Paraffin-embedded tissue sections were stained with hematoxylin and eosin and PAS for glycogen content, and they were stained immunohistochemically for placental-like alkaline phosphatase, α-fetoprotein, human chorionic gonadotropin, cytokeratins 8 and 18, and vimentin. The tumors were classified according to the World Health Organization classification for testicular tumors (Mostofi and Price, 1973). In addition, an enzyme-histochemical detection of alkaline phos-

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phatase reactivity was applied, as reported previously (Mosselman et al., 1996).

CGH

High-molecular-weight DNA was isolated from histologically checked samples (test DNA) and from peripheral blood of a normal male (reference DNA) by using standard procedures (Sambrook et al., 1989). The CGH procedure was based on the protocol described by Kallioniemi et al. (1994) with a few modifications. Briefly, test DNA was direct labeled with lissamine-dUTP (NEN Life Science Products, Brussels, Belgium), and control male DNA was labeled with digoxigenin-dUTP (Boehringer Mannheim, Mannheim, Germany). Two hundred ng of each labeled DNA and 10 μg of Cot-1 DNA were hybridized to normal male metaphases. Test DNA was detected with one layer of antidigoxigenin-fluorescein isothiocyanate (FITC; Boehringer Mannheim), and slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI), in an antifade solution (VectaShield; Vector Laboratories, Burlingame, CA). Digital images were collected by using an epifluorescence microscope (DM; Leica, Wetzlar, Germany) equipped with a triple band-pass beam splitter and emission filters (P-1 filter set; Chroma Technology, Brattleboro, VT) and analyzed by using QUIPS XL software from Vysis (Downers Grove, IL). Losses of DNA sequences were defined as chromosomal regions where the average green-to-red ratio and its 95% confidence interval were below 0.8, whereas gains were above 1.2. These threshold values were based on measurements from a series of five controls.

Cytogenetic Analysis

Representative parts of three out of the four fresh spermatocytic seminomas were processed to produce metaphase spreads directly after surgical removal, as described previously (for review, see Van Echten et al., 1995b). The chromosomal constitutions were described according to the recommendations of the ISCN (1995).

FISH

Centromere-specific probes were used to ascertain the chromosome copy number and evaluate the CGH results in the tumor designated spermatocytic seminoma 2 (SS2). The probes used were PUC1.77 (chromosome 1; Cooke and Hindley, 1979), pα12H8 (chromosome 12; Looijenga et al., 1990), D15Z1 (chromosome 15; Higgins et al., 1985), p18.4 (chromosome 18; Devilee et al., 1986), pBamX (X chromosome; Willard et al., 1983), and a probe for the Y chromosome (Wolfe et al., 1985). Probes were labeled by using nick translation either directly, with fluorochromes (lissamine-dUTP or FITC-dUTP; NEN Life Science Products), or indirectly, with biotin-dUTP (Sigma, St. Louis, MO) and were then detected with one layer of streptavidin-FITC (Vector Laboratories). Hybridizations were performed by using combinations of two probes in different colors, i.e., chromosomes 1 and 15, chromosomes 12 and 15, chromosomes 18 and 15, and the X and Y chromosomes. Target slide preparation from frozen material, in situ hybridization, and posthybridization washes were performed as described previously (Fechner et al., 1994; Rosenberg et al., 1994).

RESULTS

At the time of diagnosis, the patients were 46, 48, 48, and 83 years of age for SS1–SS4, respectively. SS2 and SS3 were from a bilateral case, and both tumors were clinically manifest within 6 months. None of the patients had cryptorchidism. The diagnosis of spermatocytic seminoma, which was made initially on a frozen hematoxylin and eosin-stained tissue sections, was supported by the negative results of enzymatic alkaline-phosphatase reactivity. In each case, the diagnosis was confirmed also by morphological and immunohistochemical analysis of paraffin-embedded sections. Three different cell types could be identified in each case: small, intermediate, and large cells, of which the intermediate cells were most abundant. No expression of α-fetoprotein, human chorionic gonadotropin, vimentin, or glycogen was detected, whereas some placental-like alkaline phosphatase-expressing cells were found in all four cases, and cytokeratin-positive cells were found in two cases (SS2 and SS4; not shown). Testicular parenchyma was present adjacent to all tumors and lacked carcinoma in situ, which is the precursor of testicular seminomas and nonseminomas (Skakkebæk, 1972). This was determined by morphological criteria, immunohistochemical analysis for expression of placenta-like alkaline phosphatase, and enzymatic staining for alkaline-phosphatase reactivity. However, intratubular spermatocytic seminoma, which is supposed to be the precursor lesion of this cancer (Scully, 1961; Talerman, 1980), was found in two cases (SS1 and SS2; not shown). No differences in immunohistochemical staining patterns were observed between the paraffin-embedded and snap-frozen samples, as described previously (Matoska et al., 1988).

CGH was performed by using DNA from representative snap-frozen tissue sections from each case

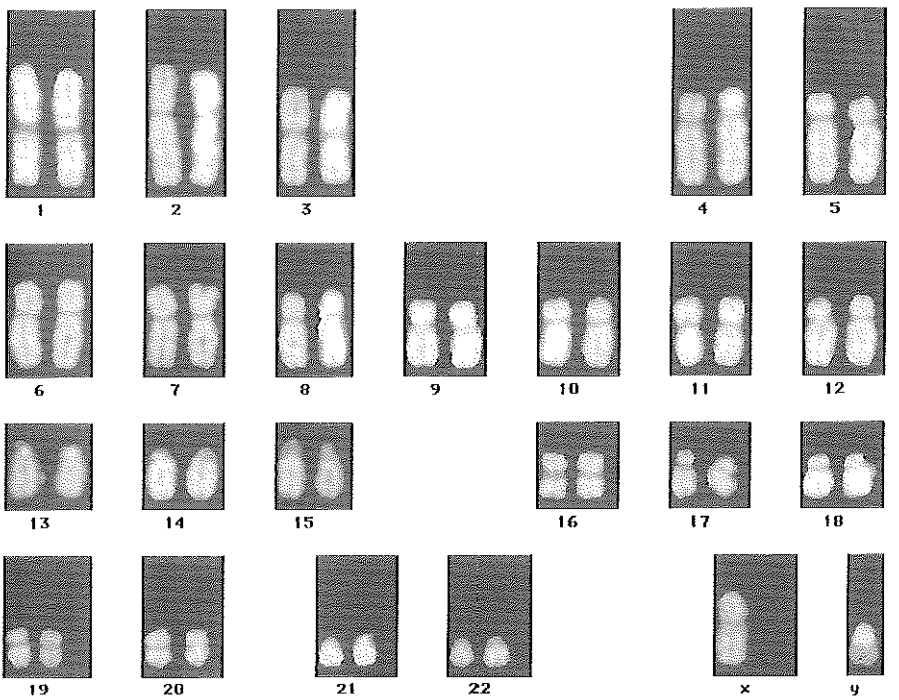

Figure 1. Results of comparative genomic hybridization of spermatocytic seminoma 2 (SS2) showing a representative example of a digital image of a normal metaphase spread (46,XY) hybridized with DNA isolated from the tumor (detected in green) and reference DNA [46,XY] (detected in red).

that had been checked histologically for the presence of at least 80% tumor cells. A representative pseudocolor CGH image from one of the tumors, SS2, is shown in Figure 1. The chromosomal imbalances detected are summarized for each tumor in Figure 2. Excluding the Y chromosome, 23 imbalances were found in total. All included full chromosome gains or losses, with one exception: overrepresentation of 1q in SS4. Some imbalances were detected only once, i.e., gain of chromosomes 5, 6, and 21 and loss of chromosomes 7, 19, and X. Others were found in at least two cases, in particular, gain of chromosomes 1(q), 9, 18, and 20 and loss of chromosomes 13, 15, and 22, of which the aberrations of chromosomes 13, 15, and 18 were present only in the bilateral cases. In total, the bilateral cases showed seven gains of (five different) chromosomes and also seven losses of (five different) chromosomes. Eight of the 14 imbalances (57%) were detected in both cases. Gain of chromosome 9 was present in all spermatocytic seminomas

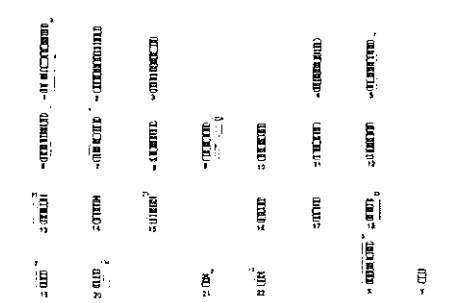

Figure 2. Summary of chromosomal imbalances detected by comparative genomic hybridization in the four spermatocytic seminomas studied. Lines on the left and right sides of the ideograms indicate under- and over-representation of chromosome material, respectively. The numbers on top of each line refer to the case analyzed. Note that SS2 and SS3 are bilateral.

tested, whereas gain of chromosomes 1 and 20 and loss of chromosome 22 were found in at least two independent cases. Other than involvement of

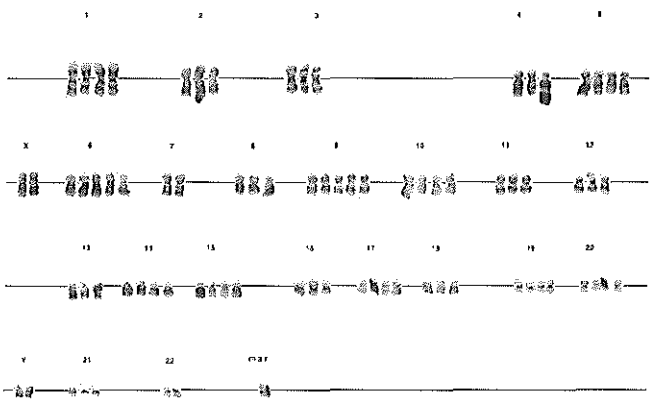

Figure 3. Representative karyotype of spermatocytic seminoma (SS1). For composite karyotype description, see Results.

chromosome 9, seven of the 13 chromosomes showed imbalances in two or more cases, and all presented either gain or loss.

In an attempt to produce metaphase spreads that were suitable for cytogenetic analysis, three of the four spermatocytic seminomas (cases SS1, SS2, and SS3) were processed directly after orchidectomy. Despite the high mitotic indices, as demonstrated by histological examination (not shown), the attempt was successful only for SS1, in which 22 metaphase spreads could be analyzed completely. A representative karyotype is shown in Figure 3. In agreement with the CGH results (see above), mainly numerical aberrations were detected. The chromosome number ranged from 74–83, which corresponds to a borderline ploidy between triploid and tetraploid. The composite karyotype was 74~83<3n>, XY,add(X)(p11)[4],+Y,+1, add (4)(p15.1)[8], +5,+6, +add(6)(q21)[8], -7,+9,+9, +10, inv(10)(q22q24)X2, del(12)(p11.2p12)[5], +14, +17, +19, +20, -22,+mar [cp22]. More than four copies of chromosomes 5, 6, 9, 17, and 20 were found, and fewer than 2.5 copies of chromosomes 7, 10, and X were found: By using CGH, these copies were to be over-represented and under-represented, respectively, with the exception of chromosomes 10, 17, and X. The undetected loss of the X chromosome is explained by the use of male DNA as a reference. The structural (nonbalanced) aberrations that were identified by cytogenetic analysis probably were not detected by CGH because of the fact that only a minority of the metaphases studied contained these aberrations. Although 22 metaphases were studied, the structural aberrations were found in eight or fewer.

Bicolor FISH with centromeric, region-specific probes was performed on isolated nuclei from frozen material from case SS2 (one of the bilateral cases). Because CGH indicated that only complete chromosomes showed imbalances in this particular tumor, centromere-specific probes were suitable to determine the copy numbers of chromosomes. The centromeres investigated included chromosomes that showed no imbalances in CGH (chromosomes 1 and 12), one that showed under-representation (chromosome 15), and one that showed over-representation (chromosome 18) along with sequences for the centromeres of chromosomes X (which also showed no imbalances with CGH) and Y (which showed amplification with CGH both by profile and by visual evaluation). Most nuclei showed two to four signals for the centromere of chromosomes 1 and 12. However, almost no nuclei were found that contained three copies of chromosome 12. The majority of nuclei contained one or two signals for chromosome 15 and four or five signals for chromosome 18. Most nuclei showed one or two copies of the X chromosome and two to four copies of the Y chromosome. These results are in overall agreement with the CGH data found (see above). The bicolor FISH approach illustrated that different subpopulations of tumor cells can be distinguished within this spermatocytic seminoma (not shown). All nuclei containing one X chromosome also contained one Y chromosome, and most of the nuclei containing two copies of the X

chromosome also showed two copies of the Y chromosome. However, the majority of nuclei with three copies of the X chromosome contained two copies of the Y chromosome, which was also found for nuclei that contained four copies of the X chromosome. Only nuclei with either one or two copies of chromosome 15 were identified, whereas the copy numbers of chromosomes 1, 12, and 18 varied between two and four, two and seven, and one and five, respectively.

DISCUSSION

The lack in the literature of cytogenetic data on spermatocytic seminomas is likely to be due to the rarity of these tumors. In a period of about 6 consecutive years, we collected a total of 357 fresh testicular germ cell tumors from adolescent and adult patients, i.e., seminomas and nonseminomas, in the Southwestern part of the Netherlands. In the same period, the four fresh spermatocytic seminomas described here were collected.

The morphological and (immuno)histochemical data from the spermatocytic seminomas included in this study were in agreement with earlier findings (Talerman, 1980; Matoska et al., 1988; Dekker et al., 1992; Burke and Mostofi, 1993; Cummings et al., 1994; Eble, 1994). However, a number of relevant observations were made on the chromosomal constitution of the spermatocytic seminomas by using CGH supported by cytogenetic analysis in one case and supported by FISH in another case. The majority of chromosomal aberrations detected were gains and losses of full chromosomes, indicating missegregation of chromosomes as the main mechanism for producing chromosomal aberrations in spermatocytic seminomas. The fact that structural chromosomal aberrations are rare in spermatocytic seminomas might be related to their benign behavior (Talerman, 1980; Talerman et al., 1984; Burke and Mostofi, 1993). Therefore, it may be interesting to study the chromosomal constitution of the sarcomatous elements that are sometimes found in spermatocytic seminomas, predicting a poor clinical outcome (Matoska et al., 1988; Burke and Mostofi, 1993; Eble, 1994). Other than the pattern of almost exclusively numerical aberrations, gain of chromosome 9 was found in all four spermatocytic seminomas, which, therefore, seems to be important in the development of this cancer. It is of interest that this particular chromosome is usually under-represented in testicular seminomas and nonseminomas (Mostert et al., 1996; for review, see Van Echten et al., 1995a). These tumors also originate from germ cells but from an earlier stage of maturation, most likely primordial germ cells (Skakkebæk, 1972; Jørgensen et al., 1995). Among the other imbalances found in the spermatocytic seminomas tested, no chromosome that was found to be under-represented in one sample was found to be over-represented in another, and vice versa. This indicates that gains and losses of chromosomes in spermatocytic seminomas are nonrandom. These findings, together with the fact that gain of 12p sequences is characteristic for testicular seminomas and nonseminomas (Van Echten et al., 1995a; Korn et al., 1996; Mostert et al., 1996) but are not found in spermatocytic seminomas (this report), add the cytogenetic differences between these two types of germ cell-derived tumors of the adult testis to the differences already established at other levels.

Morphologically, spermatocytic seminomas are composed of three types of cells: small, intermediate, and large (Burke and Mostofi, 1993; Cummings et al., 1994; Looijenga et al., 1994; this study; for review, see Eble, 1994). Although cytogenetic analysis did not demonstrate any heterogeneity, most likely due to selection of a subpopulation of cells in mitosis at the time of handling, the results obtained by using bicolor FISH indeed showed the presence of cell populations with different chromosomal constitutions, despite the fact that CGH revealed no imbalances for chromosomes 1, 12, X, and Y. These chromosomes constitute a good parameter with which to estimate the ploidy of the tumor cells. Although distributions of these chromosomes differ among each other, the chromosomes that were found to be "balanced" by CGH presented a distribution between two and four copies, with an average of near trisomic. The distribution of the sex chromosomes suggests that at least diploid (XY), tetraploid (XXYY), and a more heterogeneous population of hypertetraploid (XXXYY and XXXXYY) cells are present. Because almost all cells contained one or two copies of chromosome 15, we conclude that loss of chromosome 15 is an early event in the pathogenesis of this spermatocytic seminoma, i.e., before generation of the hyperdiploid cells. In contrast, a more heterogeneous copy number distribution was found for chromosomes 1, 12, and 18, indicating that more complex mechanisms of gains and losses in addition to simple polyploidization of the cells affect these chromosomes. Although these data support the morphological and ploidy analysis by showing the presence of different populations of cells in spermatocytic seminomas, no conclusions can be drawn regarding the relationship between

Chapter 4

Fluorescence *in situ* hybridisation-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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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 (pα12H8), 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 pα12H8- 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 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

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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_2) 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 1–5s at room temperature with 0.25% trypsin (Difco, Brunschwig, 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 (Brunschwig 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 pα12H8 [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 µl 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/µl for pα12H8 and 20 ng/µl 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-denaturated (100°C for 3 min) pα12H8 was added to the hybmix after preannealing of YAC#5. The denaturated probe mix was added to denaturated 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 summarized 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 (Jackons, Immuno Research,

Table 1 Modal composite karyotypes of the testicular germ cell tumor derived cell lines studied[a]

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(11;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;?;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;?;11)(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]

[a]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 pα12H8 and YAC#5 was performed. In this approach, pα12H8 was labeled with digoxigenin (detected with TRITC) and the YAC with biotin (detected with FITC). In addition, pα12H8 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. 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 pα12H8-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 pα12H8 was used, as illustrated by Fig. 1E, which shows nucleus of NT2 hybridized simultaneously with pα12H8 and YAC#5. The appearance of the different signals indicates the presence of three normal chromosomes 12 and two i(12p)s. The pα12H8-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

Table 2 Summary of the single and double FISH results using the centromere-specific probe pα12H8 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/
	pα12H8	pα12H8	YAC	pα12H8
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 pα12H8-derived signals for each cell line is shown.

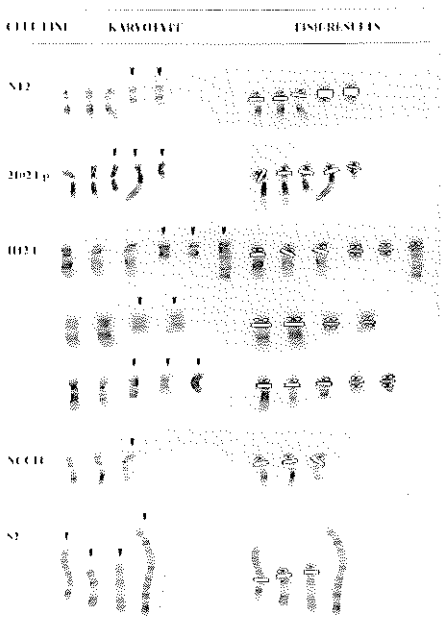

Figure 1 Fluorescence in situ hybridization (FISH) results in chromosome 12 and derivatives (arrow) with the centromeric probe pα12H8 (indicated by a block) and yeast artificial chromosome (YAC)#5 (circles).

spreads. Because of the involvement of the centromere of chromosome 12 in this translocation, it must be reclassified as der(12)t(3;12)(q11;q10).

In metaphase spreads of 2102Ep, NCCIT, and S2, similar numbers of centromeric regions were detected with the biotinylated and digoxigenin-labeled pα12H8 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 pα12H8 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 pα12H8 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 pα12H8 of ≥ 1.3. The cell lines with no i(12p) (NCCIT and S2) had a ratio of ≤ 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

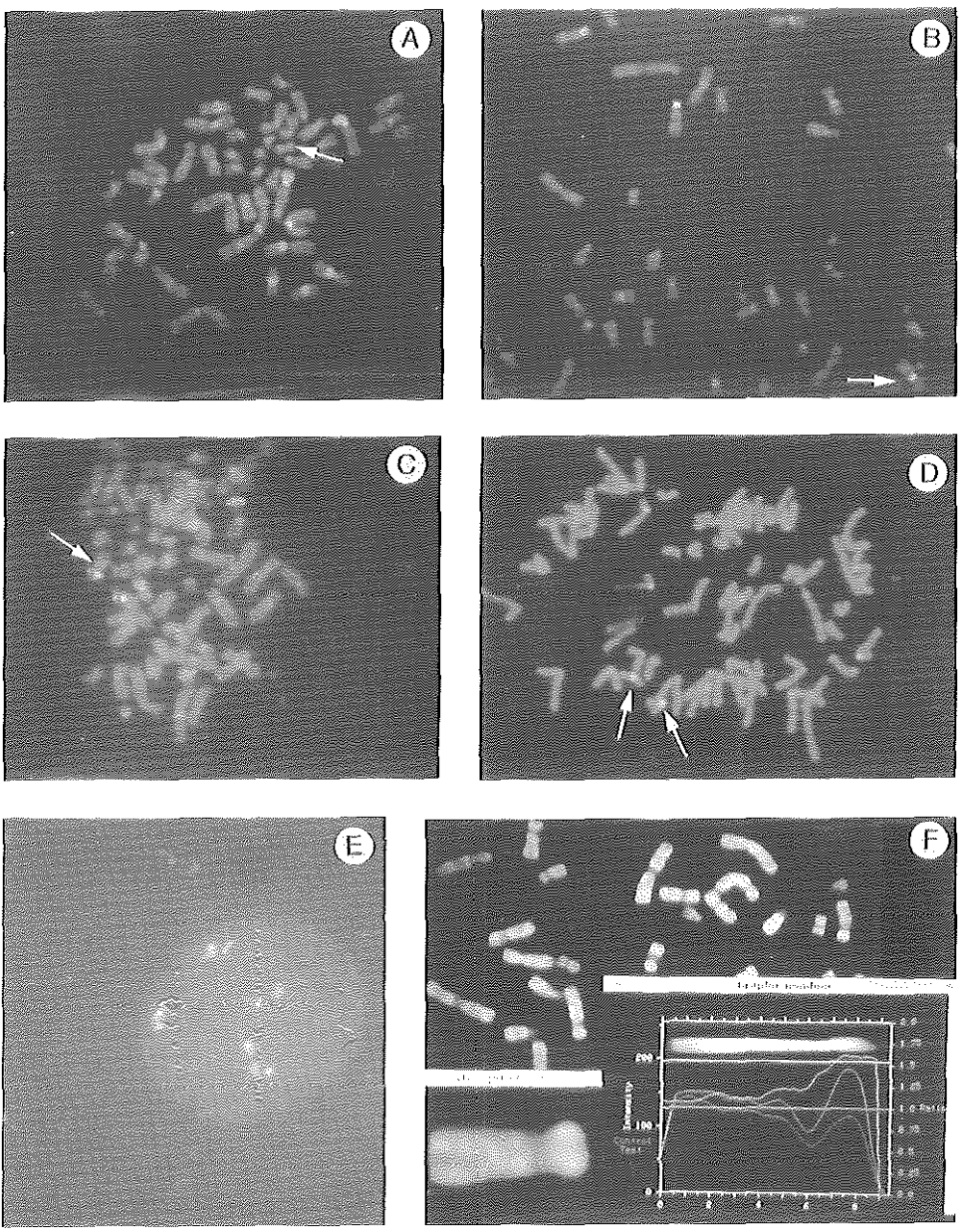

Figure 2 (A) Double fluorescence in situ hybridization (FISH) of a metaphase spread of S2 with the centromere-specific probe pα12H8 (tetramethyl-rodamine isothiocyanate TRITC; red signal) and 12p paint (FITC, yellow signal), and DAPI as counterstaining of the chromosomes; No 12p- or centromere-derived 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 consititution (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 (≤ 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)t(4;12)(p13;q11) (lower right). (B) Double FISH with pα12H8 (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 pα12H8 (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 pα12H8 (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 pα12H8 (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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Chapter 5

Comparative genomic hybridisation of germ cell tumors of the adult testis: confirmation of karyotypic findings and identification of a 12p-amplicon

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Comparative Genomic Hybridization of Germ Cell Tumors of the Adult Testis: Confirmation of Karyotypic Findings and Identification of a 12p-Amplicon

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ABSTRACT: *Comparative genomic hybridization (CGH) was carried out on 15 primary testicular germ cell tumors (TGCT) of adolescents and adults and two metastatic residual tumors after chemotherapeutic treatment. The results were compared with karyotypic data obtained form the same tumor specimens after direct harvesting of metaphases or short-term in vitro culture. Both techniques revealed that the most consistent abnormality in primary TGCT is gain of 12p-sequences. Although in most cases overrepresentation of the complete short arm was observed, CGH revealed a specific amplification of 12p11.1-p12.1 region in two independent primary tumors. In addition, loss of (parts of) chromosome 13 (always involving q31-qter), and gain of (parts of) chromosome 7 (mostly involving q11), (parts of) chromosome 8, and the X chromosome were detected in more than 25% of the tumors by this latter technique. Loss of 6q15-q21 in both residual tumors analyzed may suggest a role for this anomaly in acquired resistance to chemotherapeutic treatment.*

Overall, the CGH analyses confirmed gains and losses of certain chromosomal regions in TGCT as observed by karyotyping, and thus support their role in the development of these neoplasms. The amplification of a restricted region of 12p in primary TGCT confirms and extends our previous observations and, as such, represents an important step forward in the identification of gene(s) on 12p relevant for the pathogenesis of these tumors.

INTRODUCTION

The most common cancer in the caucasian young adult male population is the germ cell tumor of the testis (TGCT) [1, for review]. Because its incidence is still increasing, and effective treatment in its preinvasive stage is possible [2], understanding of the mechanisms involved in the etiology of this neoplasm may have important implications for the development of early detection strategies. In spite of its technical limitations in the study of solid tumors in general, karyotyping has been highly informative in the detection of chromosomal aberrations in TGCT [3–5]. In accordance with results of total DNA content analysis [6, 7], TGCT are found to be aneuploid, around the triploid range. Besides consistent over- and underrepresentations of some (parts of) chromosomes, the only recurrent structural abnormality is the isochromosome 12p [i(12p)] [5], first reported in TGCT by Atkin and Baker in 1983 [8]. This anomaly is found less frequently in seminomas (SE), composed of malignant counterparts of early germ cells, than in nonseminomatous TGCT (NS), which are caricatures of early embryonal development composed of embryonal (embryonal carcinoma (EC) and teratoma (TE)) and/or extraembryonal (yolk sac (YS) and choriocarcinoma (CH)) components [9, 10]. Fluorescence in situ hybridization (FISH) using region-specific probes showed that also i(12p)-negative TGCT invariably exhibit overrepresentations of 12p-sequences [11, 12]. Because conventional cytogenetic information is derived from mitotic cells, such data may be biased due to selection of

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subpopulations of cells by the methods of direct harvesting or short-term in vitro culture. This has recently been suggested for SE with respect to the presence of i(12p) [13]. Also the presence of markers of which, by definition, the chromosomal origin can not be determined, may hamper the identification of certain chromosomal regions important for the development of this cancer.

Comparative genomic hybridization (CGH) allows the detection of DNA copy number changes in relatively small amounts of tumor DNA [14]. Because no metaphase spreads are required CGH can be applied regardless of mitotic activity, thereby excluding in vivo or in vitro selection. In addition, CGH offers the possibility of revealing regions of amplification or deletion that are not identifiable via karyotyping.

We analyzed a series of 15 primary TGCT (including both SE and NS) and two metastatic residual tumors after chemotherapeutic treatment using both karyotyping and CGH. The metaphase spreads were isolated via direct harvesting of the SE and after short-term in vitro culture of the NS. Similarities and differences in the chromosomal constitution of the various tumor samples as detected by both techniques are evaluated.

MATERIALS AND METHODS

Patient samples

Fifteen TGCTs collected from collaborating hospitals in the Western part of the Netherlands were used in this study. The tumors were classified according to the World Health Organization (WHO) recommendations [9], as described [6], and identified as six SE, seven NS, and two combined tumors (CT) [15]. The tumors were surgically removed before the patient was treated with chemotherapy and/or irradiation. In addition, two metastatic residual tumors, one mature TE (2101) and one EC (5695), were studied. Representative parts of tumor component(s) were snap frozen in liquid nitrogen (for DNA isolation) and fixed in 4% buffered formalin for paraffin embedding (for histological classification).

Slide Preparation and Karyotyping

Metaphase spreads of SE and NS were prepared according to standard procedures. Briefly, the mitotic cells of the SE were, after mechanical dissociation of the tumor, directly harvested in the presence of Colcemid (Life Technologies, New York, USA). The cells were subsequently swollen in hypotonic KCl solution and fixed with methanol-acetic acid (3:1). The NS were enzymatically digested using collagenase and cultured in vitro for a few days, after which they were treated as reported before [16, 17].

For karyotyping, air-dried preparations were banded using pancreatin (Sigma, St. Louis, USA) as reported [18]. The chromosomal constitutions were described according to the recommendations of the ISCN 1991 [19], and calculated on the basis of a triploid DNA content, to stress the over- and underrepresentations of (parts of) chromosomes relative to the triploid situation. Although the NS were randomly selected, five i(12p)-negative SE were chosen for this survey (see Table 1). Such tumors may be instrumental in the delineation of the critical region of overrepresentation of the short arm of chromosome 12, as was suggested recently through the analysis of a metastasis of a SE [12].

Comparative Genomic Hybridization and Interpretation

For CGH, metaphase spreads were prepared using standard procedures (see above) from in vitro phytohemagglutinin-stimulated peripheral blood lymphocyte cultures of a healthy male (46,XY). High molecular weight DNA of the tumor as well as control DNAs (from peripheral blood cells of healthy males), were isolated using standard procedures [20]. CGH was performed as described before [12, 21–23]. To exclude false positive and false negative observations, DNAs obtained from 10 healthy individuals were analyzed as described for the tumors. Regions that showed variations in centromeric and heterochromatic regions, p-arms of acrocentric chromosomes and telomeric regions, were omitted from analysis.

For each hybridization, 400 ng digoxigenin (11-dUTP [Boehringer Mannheim, GR]) labeled tumor DNA and a similar amount of biotinylated (14-dATP [GIBCO-BRL]) control DNA were used. Eighty μg of COT-1 DNA (Life Technologies) was added as competitor DNA to reduce background signal due to repetitive sequences. After 2–4 days of incubation under a coverslip in a moist chamber at 37°C, the slides were washed according to regular FISH protocols [24]. Visualization of the hybridized DNAs was achieved with fluorescein isothiocyanate (FITC) conjugated sheep-anti-digoxigenin (Boehringer) and pentamethine cyanine dye isothiocyanate (CY3) conjugated avidin (Jacksons ImmunoResearch Laboratories, West Grove, USA). The results were evaluated using a Zeiss Axiophot epifluorescence microscope, equipped with a Photometrics high-performance CH250/A cooled CCD-camera (Photometrics, Tucson, AZ) interfaced onto a Macintosh Quadra 950 computer using the comparative genomic hybridization applications within the Oncor-Image FISH software package (Oncor, Gaithersburg, MD). These applications allow a pseudocolor reproduction of fluorescence ratios of CY3 (reference DNA) and fluorescein (tumor DNA).

All TGCTs were analyzed assuming no host cell contamination, i.e., instrument setting of 100% tumor. Because SE are known to contain variable amounts of infiltrating lymphocytes [9], these were also analyzed assuming 50% host cells [25]. For each tumor, five metaphase spreads, with similar imbalances on both chromatids, were interpreted according to previously published criteria [22, 23, 26]. Digital analysis allowed representation of the results as fluorescence intensity profiles for each fluorochrome along the chromosome. Photographic images were made using a Tektronics SDXII color dye sublimation printer.

RESULTS

The cytogenetic data of the primary TGCTs and the residual tumors are summarized in Table 1 as modal representative karyotypes. All showed an aneuploid DNA content around the triploid range with multiple numerical and structural aberrations. The modal number of short and long

Table 1 Modal representative karyotypes of the testicular germ cell tumors of adolescents and adults (TGCTs), based on a triploid DNA content (the aberrations also detected by a comparative genomic hybridization are underlined)

Histology	Case	Chromosomal Constitution
Seminoma		
	4255	68–76,XXY,add(1)(q12),+dic(1;12)(p13;q22),−5,+?del(6)(q21q23),+7,+8,+8,−13,+14,+15,−18,+21,−22,+1−7mar[cp11]
	1665	57–59,X,der(X)del(X)(p22)t(X;12)(q23;q12),−Y,add(1)(q24),+add(1)(p13),−2,dic(3;22)(p11;p11),i(3)(p10),+i(3)(q10),−4,dic(4;13)(q35;p12),−5,−7,+8,−9,−10,−11,add(12)(q24.1),+i(12)(p10),−13,−13,dic(13;19)(p11;q11),−15,der(15)t(15;15)(p13;q11),−16,−17,−18,der(20)t(1;2;20;?)(q25q44;q32;q12;?),−21,add(21)(p12),der(21)t(21;21)(p12;q11.1),der(22)t(7;22)(q11;p12),+mar1,+mar2,+mar3,+1−3mar[cp7]
	5718	69–74,XXY,der(1)t(1;16)(p12;p11.2),add(3)(q24),−4,−5,+del(7)(p12),+der(8)t(8;12)(q10;p11)x2,−9,add(9)(q34),der(9)t(3;9)(q13;p24),−11,+12,−13,−15,−17,add(17)(p11.2),−18,add(18)(q22),add(19)(p13.2),+21,+21,+21,+22,+der(?)t(?;12)(?;p11),+mar1,+0−2mar[cp10]
	5731	71–82,XXY,+1,+2,i(4)(q10),−5,+6,+7,+8,+add(12)(p11),−13,+14,+15,+19,−20,+21,+21,+22,+mar1,+1−6mar[cp9]
	7214	55–69,XXY,+add(1)(p22),der(2)t(2;mar1)(q37;?),add(3)(p24),der(3)t(3;9)(p24;q12),add(4)(q32),der(4)i(4)(q10)add(4)(q35),+i(4)(q10),−5,add(6)(q16)del(6)(p23),+add(6)(q27),+add(7)(p11),−9,−10,−11,add(11)(q23),+12,+add(12)(p11),−13,+14,+15,add(16)(q21),−17,−18,−18,−20,+21,+add(21)(p12),+mar1,+mar2,+mar3,+mar4,+1−6mar[cp10]
	2655	69–71,XX,−Y,+der(X)t(X;14)(q22;q11),+2,+der(3)t(1;3)(q11q27),+der(4)t(4;12;?;15)(q21;q15q22;?;q13),−5,der(5)(5;?;16)(p15.3;?;q11),+der(7)t(7;?;10)(q11.2;?;q11),+8,+8,add(9)(p22),−11,add(11)(p15),add(12)(p13),add(12)(p13),+der(12)t(12;?;15)(q22;?;q13),−13,−14,−15,−16,−17,der(17)t(7;?;17)(p13;?;q25),−18,der(20)t(14;20)(q11;p11),+21,+21,+22 [cp5]/69–71,idem,−1,+add(1)(q32),−8,+i(8)(q10) [cp4]
Nonseminoma		
	239	62–64,XY,−X,+del(1)(p34),−4,−5,add(5)(q35),+del(6)(q21),+8,−9,−10,−11,+i(12)(p10),−13,−17,−18,−22,+0–5mar [cp2]
	3035	52–56,XXY,add(1)(q11),+der(1)t(1;2)(q10;p10),−2,add(3)(p12),−4,−5,−6,i(7)(p10),−9,del(9)(q11),−10,−11,der(11)t(9;11;11)(q13;q13q25;q25),−13,add(13)(q21),+add(14)(p12),−15,−16,−18,−19,−20,−21,−21,−21,−22,−22,+mar1,+mar2,+1–4mar [cp10]
	5116	68–74,XXY,tas(1;9)(p36;q34),del(6)(q13q14),+8,−9,tas(9;17)(q34;q25)+i(12)(p10)x2,+14,−17,−18,add(18)(p11.3),−22,+0–3mar [cp10]
	6537	64–66,XX,−Y,add(1)(p32),add(1)(q31),+add(3)(p14),−5,+7,−10,−11,+add(12)(q11),?i(12)(p10)x2,−13,−14,add(15)(q24),−19,20,−21,−22,+3–6mar [cp4]
	8007	53–61,X,add(X)(q11),Y,add(1)(p36),−2,add(3)(p21),−4,−5,−6,add(9)(q21),−10,−11,+i(12)(p10),−13,−15,−18,−19,−21,−22,−22,+der(?)t(?;?;12)(p11;?;p11),+mar1,+mar2,+3–12mar [cp8]
	1862	61–67,XX,−Y,−4,+7,−9,−10,−11,add(11)(q13),+i(12)ip10)x2,−13,−14,−18,add(19)(q13),+add(19)(q13),−22,+mar1 [cp8]
	2871	56–61,XXY,add(1)(p21),−4,−5,ins(5;?)(q11;?),add(6)(q24),+8,−9,−9,−9,−10,−11,−11,−13,−14,add(14)(p12),der(16)t(9;16)(p21;p12),−18,−22,+der(?)t(?;7)(?;p13),+der(?)(11;?;12)(q12.1;?;p11),+mar1,+0–1mar [cp10]
Combined TGCTs		
Seminoma	2207	60–64,XX,−Y,−3,−4,−5,add(6)(p22),+add(7)(q22),del(8)t(8;12)(p11.2;p11.2),−9,−11,add(11)(p12),add(12)(p12),−13,−14,−16,−17,−18,+21,+21,+mar1,+mar2,+mar3,+0–1mar[cp7]/60–62,der(X)t(X;1)(q21;q12),−Y,add(3)(p24),−4,−5,−6,+i(8)(q10),−9,−9,−11,−11,+add(12)(q14),−13,−16,−17,−17,−18,add(20)(q12),+21,+22,+r,+mar1,+0–3mar [cp3]
Seminoma	3284	66–67,X,add(X)(q28),−Y,add(1)(q11),add(1)(q43),+2,−4,−5,add(5)(p15),−6,−6,ins(6;?)(q11;?),+7,+8,−9,idic(9)(p24),−11,+12,−13,+15,dic(17;?;21)(p11.2;?;p12),+der(21)t(6;21)(p11;p12),add(22)(p12),+mar1 [cp9]
Residual Tumors		
	2101	59–62,XXY,del(1)(p35),−2,add(3)(p12),−4,−5,+del(8)(p11),−9,−10,add(10)(q22),−11,−13,−14,−15,−18,−19,der(20)(12;20)(p11;p11),+21,−22,+mar1,+mar2 [cp7]
	5695	57–64,XXY,+Y,add(2)(q24),i(3;15)(p23;q24),−4,dic(4;11)(p16;p11.2),−5,add(6)(q13),+8,−9,−10,−11,−11,i(12)(p10),+i(12)(p10),−13,−14,−15,−15,−16,−18,add(19)(q13.1),+der(21)t(12;21)(p11;p11),+mar1,+mar2,+mar3,+mar4,+0–2mar [cp7]/62–63,idem,+add(4)(p16),+11 [cp3]

arms (if involving more that 50% of its length and accounting for only the long arms of acrocentric chromosomes as described by De Jong et al. [3]) for each chromosome was determined. These data, depicted in Figure 1, indicate a similar pattern of chromosomal over- and underrepresentation in SE and NS, i.e., underrepresentation of (parts of) chromosomes 4, 5, 9, 11, 13, and 18, and overrepresentation of (parts of) chromosomes 7, 8, 12, and X.

The aberrations found by CGH are illustrated in Table 2 and Figure 2 (only the results obtained from analysis of SE

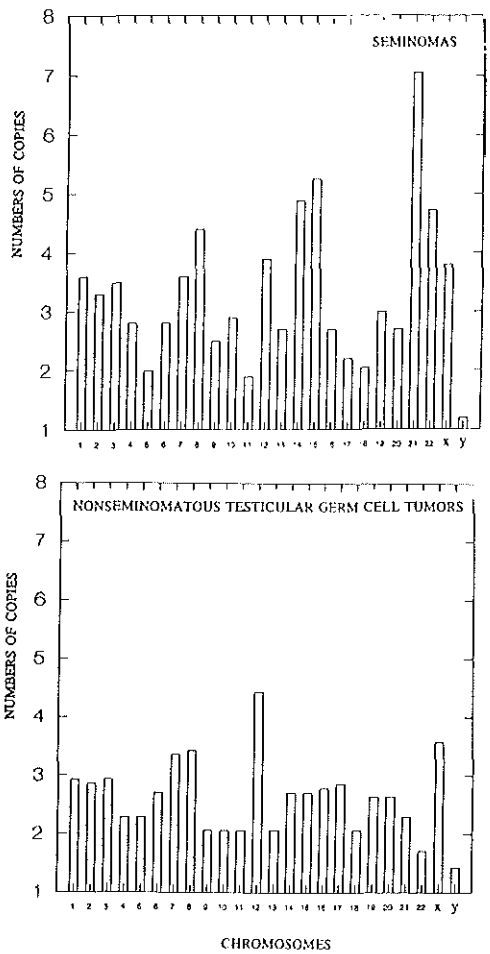

Figure 1 Schematic representation of the average copy number per chromosome for six seminomas and seven nonseminomatous testicular germ cell tumors of adolescents and adults, calculated on the basis of karyotypic results as reported before [3].

with instrument settings assuming 50% host cells are shown). Those aberrations that were also detected (partly) with karyotyping are underlined in Table 1. Within the group of the six primary SE, gain was found 14 times involving six different chromosomes, and loss six times involving three different chromosomes. For the NS (n = 7), gain was found 10 times affecting four different chromosomes and loss five times affecting four different chromosomes. No imbalances were detected by CGH for chromosomes 1, 16, 17, 20, 21, 22, and Y (these are excluded from Figure 2), whereas aberrations (of parts) of chromosomes 2, 5, 9, 18, and 19 were only found in a single primary tumor. The following regions showed aberrations in multiple cases: loss of parts of chromosome 4 (in one SE, one NS, and one SE of a CT), 11 (in one NS and a SE of a CT), and 13 (in three SE, one NS, and one SE of a CT), gain of parts of chromosome 7 (in three SE, one NS, and one NS of a CT), 8 (in three SE, one NS, and one NS of a CT), complete 12p (in three SE, six NS, and one NS of a CT), region 12p11.1-p12.1 (in one NS and both components of one CT) and X (in three SE and one NS). Some aberrations found in SE were only identified using the instrument settings assuming 50% tumor, i.e., loss of 13q31-qter in tumor 4255 and 13 in tumor 2655, and gain of 8qter in tumor 1665, 7q11 in tumor 5718, and 7, X in tumor 5731.

Although only the SE components of the CTs could be karyotyped (Table 1), both histological components were studied by CGH (Table 2). Gain of 12p11.1-p12.1 was detected in both components of tumor 2207 (illustrated in Figure 3). The ratio value of the amplicon was approximately three times higher in the SE compared to the NS (not shown). Other aberrations were found either in the SE or NS component within that CT. The other CT (3284) showed only gain of 12p in the NS component by CGH. The residual tumors showed four times gain, affected two different chromosomes, and five times loss affecting four different chromosomes. Both cases showed loss of part of the long arm of chromosome 6 (encompassing q15-q21) and gain of the complete short arm of chromosome 12.

Although most aberrations found by CGH were confirmed by karyotyping, others were unexpected, i.e., overrepresentation of region 9q21–q31 in 4255, 8q11.1-q12, and 12p11.1–p12.1 in 3035.

DISCUSSION

The series of six SE and seven NS presented here, shows a pattern of over- and underrepresentation of (parts) of chromosomes as found in a group of more than 100 TGCTs published before [5]. The recently developed technique of comparative genomic hybridization (CGH), a bicolor FISH-approach that enables detection of relative over- and underrepresentation of chromosomal regions [14], turned out to be very useful in the detection and mapping of DNA-sequence copy number changes in solid tumors [12, 14, 21–23, 27–35]. We applied this technique to a series of karyotyped TGCTs to screen for chromosomal regions showing imbalances in these tumors possibly unidentified by conventional chromosomal analysis.

Multiple cases showed gain of (parts of) chromosome 7, 8, X, and the short arm of chromosome 12, and loss of (parts of) chromosome 13 both by karyotyping and CGH. Although other losses or gains of some chromosomal regions were expected from karyotypic analysis, these were not detected by CGH. This may be partly due to the presence of chromosomal segments hidden in markers, tumor heterogeneity, the sensitivity of the CGH applied, as well as the presence of host cells in the samples (illustrated by the results in SE). However, some unexpected copy number changes were detected by CGH in our series of TGCTs. These changes mostly involved small chromosomal re-

Table 2 Gains and losses of chromosomal regions in testicular germ cell tumors of adolescents and adults detected by comparative genomic hybridization

Histology	Case	Loss	Gains
Seminoma			
	4255	13q31–qter	8, 9q21–q32
	1665		8qter, 12p
	5718		12p, X, 7q11
	5731	13	7, 8q, X
	7214	4p, 4q13–qter	4q12
	2655	13, 18	7q11, 12p.X
Nonseminoma			
	239		12p
	3035		7p, 8q10–q12, 12p11.1–p12.1
	5116		12p
	6537	19	12p
	8007		12p
	1862	4q, 11q22–qter	12p
	2871	13	12p, X
Combined tumors			
Seminoma			
	2207	4q,13q	12p11.1–p12.1
	3284	11q13–qter	2q35–qter, 6p10–p21.2
Nonseminoma			
	2207		7q11–q21, 8q21–q23, 12p11.1–p12.1
	3284		12p
Residual tumors			
	2101	6q15–q21	12p
	5695	5, 6q, 12q21–q22, 13q	8p, 12p

gions not detected with conventional karyotyping. In addition, CGH enabled us to determine the minimal regions of overlap of parts of chromosomes showing imbalances, i.e., 7q11 and 13q31-qter. These results are in line with CGH data of a paraffin embedded SE obtained after universal DNA amplification [36]. Consistent with karyotypic analysis of a large series of TGCTs ([5] and this paper), overrepresentation of 12p is the most frequent aberration found. Interestingly, CGH revealed in two i(12p)-negative tumors (one SE and both components of a CT) specific amplification of a subregion of the short arm of chromosome 12, i.e., 12p11.1–p12.1. Although this same region was recently found to be amplified in a metastasis of a testicular SE [12], we now report that this anomaly may also be encountered in primary TGCTs. A very similar observation was recently made in three additional primary TGCTs (M. Korn, personal communication).

Because karyotyping of both the SE and NS components of CTs is hard to achieve, CGH was used to compare their chromosomal constitutions. The results obtained illustrate that the SE and NS components of both cases studied here were at least partially genetically distinct. The only common aberration was the amplification of 12p11.1-p12.1 in both components of one CT. However, the ratio value in the SE was approximately three times higher than in the NS. As we recently suggested [37] these results indicate the SE and NS components of CTs might be monoclonal or biclonal in origin.

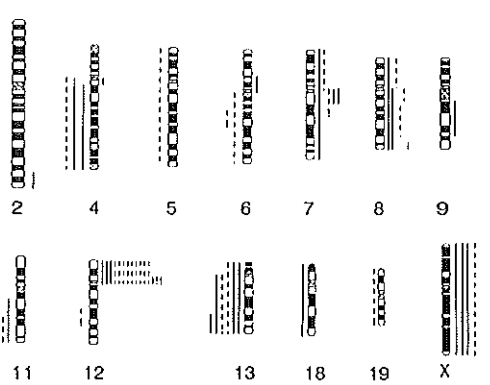

Figure 2 Schematic representation of relative loss (indicated on the left side of the chromosome) and gain (indicated on the right side of the chromosome) of chromosomal regions, detected by comparative genomic hybridization in six seminomas (marked by lines), seven nonseminomatous testicular germ cell tumors of adolescents and adults (marked by dotted lines), both components of the combined tumors (illustrated as the pure SE and NS), and two metastatic residual tumors (also indicated by dotted lines).

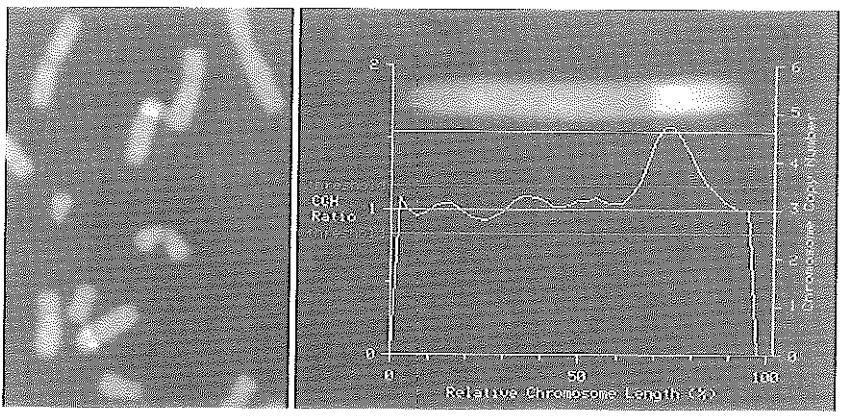

Figure 3 Example of the result of comparative genomic hybridization on chromosome 12 of the seminoma component of combined tumor 2207, showing tumor specific amplification (represented as relative overrepresentation of the green compared to the red signal), encompassing region 12p11.1-p12.1. The blue line represents the ratio profile, i.e., the red to green fluorescence intensities over the whole chromosome length.

Another interesting finding with CGH is the specific loss of 6q-sequences (involving q15–q21) in both residual tumors. This suggests that loss of gene(s) localized in this region may be involved in resistance to chemotherapeutic treatment, as recently suggested for glioblastoma multiforme [38]. Obviously, more data are required to substantiate this notion.

In conclusion, our study of CGH on a limited number of TGCTs largely confirms our karyotypic findings on a series of more than 100 tumors [5]: overrepresentation of (parts of) chromosomes 7,8,12, and X, and underrepresentation of chromosome 13. In addition, through CGH the most likely regions involved could be identified, i.e., 7q11 and 13q31–qter. The most intriguing finding, however, is the amplification of a restricted region of the short arm of chromosome 12, 12p11.1-p12.1, in different primary TGCTs. Analysis of more TGCTs using this approach will bring the isolation of the relevant gene(s) on 12p in the realm of feasibility.

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Chapter 6

Identification of the critical region of 12p over-representation in testicular germ cell tumors of adolescents and adults

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Identification of the critical region of 12p over-representation in testicular germ cell tumors of adolescents and adults

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Cytogenetically, testicular germ cell tumors of adolescents and adults (TGCTs) are characterized by gain of 12p-sequences, most often through isochromosome formation (i(12p)). Fluorescence *in situ* hybridization (FISH) has shown that i(12p))-negative TGCTs also cryptically contain extra 12p-sequences. The consistency of 12p-over-representation in all histological subtypes of TGCTs, including their preinvasive stage, suggests that gain of one or more genes on 12p is crucial in the development of this cancer. So far, studies aimed at the identification of the relevant gene(s) were based on the 'candidate-gene approach'. No convincing evidence in favor of or against a particular gene has been reported. We combined conventional karyotyping, comparative genomic hybridization, and FISH to identify TGCTs with amplifications of restricted regions of 12p. Out of 49 primary TGCTs (23 without i(12p), 13 with and 13 unknown), eight tumors (six without i(12p) and two unknown) showed amplifications corresponding to 12p11.1-p12.1. Using bicolour-FISH, physical mapping, and semi-quantitative polymerase chain reactions, the size of the shortest region of overlap of amplification (SROA) was estimated to be between 1750–3000 kb. In addition, we mapped a number of genes in and around this region. While fourteen known genes could be excluded as candidates based on their location outside this region, we demonstrate that *KRAS2*, *JAW1* and *SOX5* genes are localized within the SROA. While *KRAS2* and *JAW1* map to the proximal border of the SROA, *SOX5* maps centrally in the SROA. *KRAS2* and *JAW1* are expressed in all TGCTs, whereas one 12p amplicon-positive TGCT lacks expression of *SOX5*. The critical region of 12p over-represented in TGCTs is less than 8% of the total length of the short arm of chromosome 12. It will be helpful in the identification of the gene(s) involved in TGCT-development.

Keywords: testicular germ cell tumors; 12p; over-representation; critical region; candidate gene(s)

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Introduction

Testicular germ cell tumors of adolescents and adults (TGCTs) are the most common cancer in Caucasian males in the age between 15 and 45 years, and an increasing incidence has been reported in most European countries as well as in the USA (Adami *et al.*, 1994; Møller *et al.*, 1995; Feuer, 1995; Bergström *et al.*, 1996; Bosl *et al.*, 1997). Histologically and clinically they can be divided into two main groups, the seminomas (SE) and the nonseminomatous-TGCTs (NS) (Mostofi *et al.*, 1987). Both types of TGCTs originate from a common precursor, known as carcinoma *in situ* (Skakkebæk *et al.*, 1987) or intratubular germ cell neoplasia (Stamp and Jacobsen, 1995). A large number of TGCTs, both primary and metastatic, of various histologies have been studied for their chromosomal constitution by different investigators, including ourselves (Rodriquez *et al.*, 1993a; Van Echten *et al.*, 1995; Sandberg *et al.*, 1996, for review). Besides non-random patterns of over- and underrepresentation of (parts of) chromosomes, gain of 12p is a consistent finding. In the majority of TGCTs tested this was due to the presence of one or more copies of isochromosome 12p [i(12p)]. In addition, it was found using fluorescence *in situ* hybridization (FISH) that TGCTs without i(12p) always show over-representation of 12p-sequences cryptically hidden in the genome (Suijkerbuijk *et al.*, 1993; Rodriquez *et al.*, 1993b; Smolarek *et al.*, 1995). We showed that i(12p) may also be present in CIS (Vos *et al.*, 1990), indicating that over-representation of 12p-sequences is probably a relatively early event in the pathogenesis of this cancer, not related to invasive growth. We confirmed this by numerical analysis on tissue sections using *in situ* hybridization (Looijenga *et al.*, 1993; Gillis *et al.*, 1994).

Although more than 14 years have passed since the first publication of i(12p) as a specific chromosomal abnormality for TGCTs (Atkin and Baker, 1983), ideas on the critical target gene(s) still remain speculative. This can be explained by the fact that most TGCTs show over-representation of the complete short arm of chromosome 12, encompassing approximately 40 Megabases (Mb) and containing theoretically between 850 and 1400 genes (Marynen and Kucherlapati, 1996). Therefore, alternative approaches are required to identify the gene(s) on 12p of which copy-gain is

beneficial for the development of TGCTs. One possible strategy was initiated by our previous identification of a metastatic SE showing amplification of a restricted region of 12p (Suijkerbuijk *et al.*, 1994). More recently, we and others demonstrated via comparative genomic hybridization (CGH), a technique developed to identify relative (sub-) chromosomal gains and losses (Kallioniemi *et al.*, 1992), that also a certain percentage of primary TGCTs shows amplification of a restricted region of 12p, cytogenetically similar to the one observed before (Mostert *et al.*, 1996a; Korn *et al.*, 1996). This finding of a restricted 12p-amplification allows identification of relevant genes using a direct approach.

To determine the shortest region of overlap of amplification (SROA) of 12p in TGCTs we combined conventional karyotyping, CGH, (bicolour-)FISH, physical mapping, and semi-quantitative polymerase chain reaction (PCR) analyses. Identification of such a SROA allows the exclusion of a number of known genes on 12p as candidates, and will facilitate a more direct approach to identify novel genes on 12p critical to the pathogenesis of this cancer.

Results

Detection of 12p-amplification

To identify cases with a restricted amplified region of 12p, we used CGH to screen a series of 49 primary TGCTs, including 26 SE and 23 NS, of which 23 lacked i(12p) by conventional karyotyping (13 SE and 10 NS). In 13 tumors i(12p) was identified and 13 tumors could not be analysed due to lack of metaphases. Eight tumors (six lacking i(12p) and two unknown) showed amplification of a part of 12p detectable by CGH, identified as region 12p11.1-p12.1, similar to the previously reported case of a metastatic SE (Suijkerbuijk *et al.*, 1994). Four tumors were classified as SE (T2655, T2814, T8763 and T202) and four as NS (T10077, T3035, T2207 and T60). Tumors T2207 and T60 are NS with a SE-component, socalled combined tumors according to the British classification (Pugh, 1976). The amplification was found in both SE components, and in only one of the NS components (not shown).

Six of these tumors with amplification (T2655, T2814, T8763, T10077, T3035 and T2207) were karyotyped successfully. The metaphases were further analysed by FISH with a Yeast Artificial Chromosome probe, known as YAC#5 which was formerly mapped to region 12p11.2-p12.1 (Suijkerbuijk *et al.*, 1994). This probe was used since it allowed the detection of gain of 12p-sequences in all TGCTs tested so far (unpublished observations). It was found that the amplifications were due to the presence of additional 12p-sequences in add(12)(p13) or der(12)t(12;?;hsr)(p13;?;?) in T10077, add(12)(p13) in T2655, add(12)(p12) in T2814, der(6;?17)(q10;q10)add(6)(q21) and a marker (which was cytogenetically identified as an i(8)(q10)) in T8763 (not shown). In the remaining two tumors there were no metaphases suitable for FISH.

All eight TGCTs with a proven restricted amplification of 12p by CGH were studied by FISH using YAC#5 on interphase nuclei. This probe showed between 15–30 hybridization signals per nucleus in these tumors as reported before in other TGCTs (Suijkerbuijk *et al.*, 1994; Mostert *et al.*, 1996b; Korn *et al.*, 1996). Circumstantial evidence that YAC#5 indeed maps within (or at least close to) the critically amplified region was obtained by combining CGH on tumor T2207 with FISH of YAC#5: YAC#5 hybridization co-incided exactly with the amplified 12p-region, both in metaphase spreads and interphase nuclei (not shown). Of the samples T2655 and T2207 suitable material for further FISH analysis was lacking, because only a small part of tumor cell nuclei contained the 12p-amplification. The 12p-amplicon negative nuclei in these particular tumors however, also contained extra 12p sequences identifiable with FISH, although in a lower total copy number. In which form these sequences were present could not be determined due to lack of metaphases. Using FISH with YAC#5 in combination with a probe specific for the centromeric region of chromosome 12 (Looijenga *et al.*, 1990) we checked the remaining 41 TGCTs for 12p-amplification. This approach showed no indications that amplification of a restricted region (containing YAC#5) was missed by the procedure followed.

Construction of a YAC-contig of 12p11.2-p12.1 region and mapping of YAC#5

YAC#5, previously mapped to 12p11.2-p12.1 (Suijkerbuijk *et al.*, 1994), was used as a starting point to generate a contig suitable to determine the SROA. The most relevant part of the contig is shown in Figure 1a. The length of the different YACs was estimated by pulsed-field gel electrophoresis. The presence or absence of STS markers was checked by PCR using STS specific primer sets on the relevant YACs. Both proximal and distal directions were analysed until a YAC was proven to be negative for the next STS. The assumed order of STS markers was in agreement with the data from the Whitehead Institute database. The seeming contradiction in length of YACs 803B2, 845D11 and 957B7, which all are positive for the same markers (see Figure 1a), is most probably due to chimerism of the YACs 803B2 and 845D11, as these YACs also score positive with markers of other chromosomes than chromosome 12. YAC#5 was placed on the contig by inter-Alu fingerprinting and by Southern blot hybridization of a YAC#5 interAlu probe (not shown). As indicated, YAC#5 was found to overlap with YACs 922D9, 928G2 and 753F12. In addition, YAC#5 was positive for STS D12S1057 by PCR, which secured YAC#5 to a fixed position on the map. Furthermore, a number of cosmids specific for different STSs within the contig were isolated (Figure 1a).

Shortest region of overlap of amplification (SROA)

To define the borders of the SROA, the six remaining 12p amplification-positive tumors were studied using bicolour-FISH, as reported before (Mostert *et al.*, 1996b). Therefore, different YACs and cosmids within the contig were hybridized simultaneously with YAC#5 on interphase nuclei isolated from the tumors. YAC#5 was visualized in green and the other probes in red. The number of signals per interphase nucleus, and

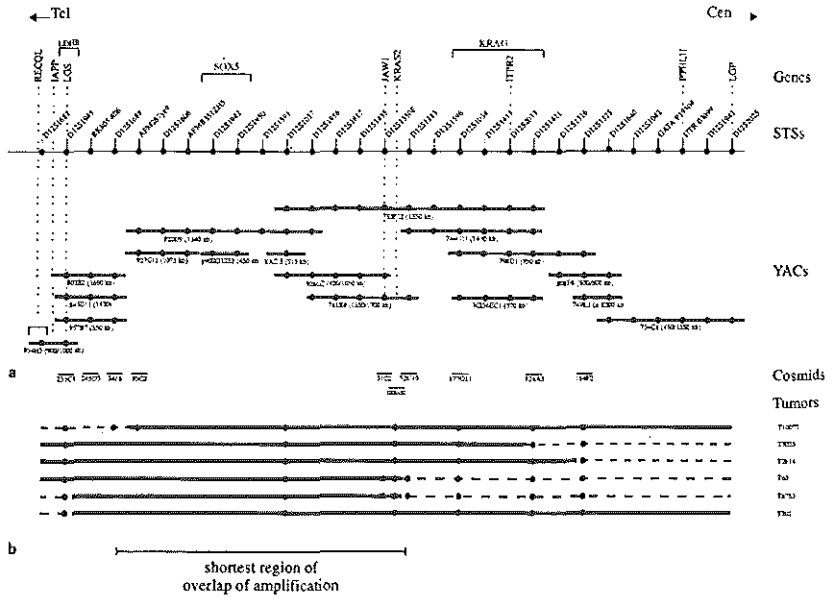

Figure 1 (a) Schematic representation of the physical map of the critical 12p11.1-p12.1 region, including the different STS markers. Positions of YACs, cosmids and genes are indicated. Note the localizations of YAC#5, *KRAS2*, *JAW1* and *SOX5*. The length of the YACs is indicated in brackets; (b) summary of the results obtained by bicolour-FISH on tumor samples using YAC#5 simultaneously with other probes from the region. The regions which are amplified in the tumors at a similar level as YAC#5 are indicated by bold lines, while the regions that are not included in the amplicons are illustrated by dotted lines. Note that the shortest region of overlap of amplification maps between the markers D12S1688 at the distal side and D12S1313 at the proximal side. The length of the YACs and cosmids are not drawn to scale

metaphase spread if available, was scored. The results are summarized in Figure 1b. None of the YACs tested showed higher numbers of signals than YAC#5, indicating that the region showing the highest level of amplification includes sequences recognized by YAC#5. For all tumors similar signal numbers were found for YAC#5 and YACs 927G11, 922D9 and 753F12. In contrast, at the telomeric side, YAC 803B2 showed significantly fewer signals than YAC#5 in T10077, whereas on the centromeric side YAC 744G11 showed significantly less signals in T60 and T8763. Due to the sizes of the different YACs used (indicated in Figure 1a), the exact borders of the amplicon could not be determined accurately. Therefore, a number of cosmids (specific for STSs localized in the putative border regions of the amplicon) were used in a similar bicolour-FISH approach as described above with YAC#5. The results are also indicated in Figure 1b and a representative example is shown in Figure 2. In the distal region, cosmid 235C1, containing STS D12S1945, showed fewer signals than YAC#5 in T10077, T8763 and T202. About 25% of the nuclei of the latter tumor showed this difference, while the majority of nuclei had similar copy numbers. FISH with cosmid 34F8, containing D12S1688, showed the same pattern as with 235C1 in T10077 while cosmid 95C2, specific for AFM267yc9, demonstrated an equal number of signals as with YAC#5. The data indicate that the distal border of the SROA maps between the STSs D12S1688 and AFM267yc9.

At the proximal side fewer signals than with YAC#5 were found in four of the tumors with cosmid 164F2 (containing STS D12S1325), in three tumors with 124A3 (containing STS D12S1411), in two tumors with both 177G11 (containing STS D12S1034) and 52C10 (containing STS D12S1313). Cosmid 31C2 (containing STS D12S1350E) showed similar signal numbers as with YAC#5. The data indicate that the proximal border of the SROA lies between D12S1350E and D12S1313.

It can be concluded that based on this set of tumors, the SROA lies between D12S1688 at the distal end and D12S1313 at the proximal end. More tumors containing smaller 12p-amplifications have to be identified and studied to further narrow the SROA.

Candidate genes and semi-quantitative (RT–PCR)

A number of genes have been mapped to the region 12p11-p13, as summarized in Table 1. These can be considered as candidates to explain gain of 12p-sequences in TGCTs. We were able to map the genes (from distal to proximal), *RECQL*, *IAPP*, *LGS*, *LDHB*, *SOX5*, *JAW1*, *KRAS2*, *KRAG*, *ITPR2*, *PTHLH* and *LGP* within the contig (Figure 1a). *LGS* (Nuttall *et al.*, 1994), *ITPR2* (Heighway *et al.*, 1996),

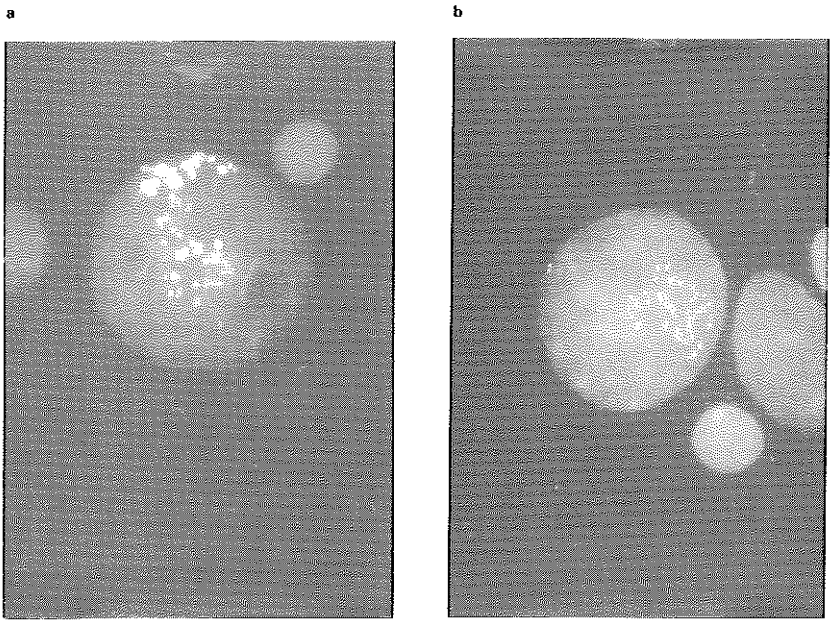

Figure 2 Example of bicolour fluorescence *in situ* hybridization on isolated nuclei from tumor T8763, showing (a) similar signal numbers using YAC#5 (detected in green) and cosmid 31C2 (containing STS D12S1350E) (detected in red) as probes; and (b) a lower signal number for cosmid 124A3, specific for STS D12S1411 (detected in red) as compared to YAC#5 (detected in green). Signals for the cosmids (in red) are larger than the signals for the YACs (in green) because of a biotinyl-tyramide amplification step used to visualize the cosmids

PTHLH (Raeymaekers *et al.*, 1995) and *LGP* (Newgard *et al.*, 1986) have been localized as part of a long term project to map all known human genes and ESTs (Krauter *et al.*, 1995, and http://www genome.wi.mit.edu). YAC ICI36EC1 was the smallest YAC found to be positive for *KRAG* by PCR, placing this gene between STS D12S1596 and D12S1316. The human DNA helicase Q1 (*RECQL*) (Seki *et al.*, 1994; Puranam, 1995), the islet amyloid polypeptide (*IAPP*) (Hoovers *et al.*, 1993) and lactate dehydrogenase isoenzyme B (*LDHB*) genes (Li *et al.*, 1988) were previously mapped to band 12p12. We were able to localize these genes to the distal part of our YAC contig (Figure 1a) and their order was determined by PCR on the different YACs.

Three genes, *SOX5*, *JAW1* and *KRAS2* were found to map within the SROA. *KRAS2* was previously mapped between markers D12S1435 and D12S1596 (Krauter *et al.*, 1995). PCR with *KRAS2* specific primers showed that proper amplification products were generated from YACs 753F12 and 761B8, while no signals were obtained using YACs 928G2 and 744G11. This observation places *KRAS2* between the markers D12S1350E and D12S1313. This localization was confirmed by Southern blot analysis using a *KRAS2* specific probe (not shown). Our data demonstrate that *KRAS2* is localized just at the proximal border of the SROA (see Figure 1). Bicolour-FISH using YAC#5 and a cosmid containing *KRAS2* showed that this region was indeed amplified at the same level as YAC#5 in all tumors. *SOX5* was previously mapped to 12p12.1 by FISH (Wunderle *et al.*, 1996). Southern blot analysis with an inter-Alu specific fragment of YAC y900G1253, which contains the *SOX5* gene (Wunderle *et al.*, 1996), showed only hybridization with YAC 922D9 (not shown). PCR with *SOX5* specific primers confirmed localization of this gene on both YAC 922D9 and y900G1253. In addition, YAC y900G1253 scored positive for STSs D12S1942 and D12S1950. Therefore, we conclude that *SOX5* maps between the markers AFMB351ZH5 and D12S1591 (illustrated in Figure 1a). A sequence homology search with markers from our contig showed 100% identity of STS D12S1350E with the *JAW1* gene.

As an additional approach to define the SROA, semi-quantitative PCR-analyses were performed on five TGCTs with a 12p-amplicon (T10077, T3035, T2814, T60 and T8763) to study differences in copy numbers of genes mapping to the region of interest: i.e., *LGS*, *KRAS2*, *KRAG* and *ITPR2* (Figure 3). Two non-12p genes were included as references (*Prog*R and *17pTEL*), which revealed similar results. No clear differences in copy numbers of the tested genes on 12p were found in TGCTs without 12p-amplicons that were used as controls (not shown). Also, no unbalances were found between the 12p-genes themselves in T2814, which is in agreement with the results of the bicolour-FISH experiments. While all 12p genes tested showed over-representation at least to some degree relative to *ProgR* and *17pTEL* in T10077, T3035 and T2814, no

gain of *KRAG* and *ITPR2* was found in T60 and T8763, again concordant with the results obtained by bicolour-FISH. This indicates that this region of 12p is not included in the amplicons in these particular

Table 1 Summary of the different genes mapped to chromosomal region 12p11-p13; their localization within the YAC-contig and the shortest region of overlap of amplification (SROA) is indicated

Gene	*Description*	*Maps to YAC*	*Maps to STS*	*Region*	*SROA*[a]	*Mapping method*[b]	*Reference(s)*
CCND2	Cyclin D2	–	–	12p13	–	Sc,Db	(Raeymakers *et al.*, 1995)
CD69	Early T-cell activation antigen	–	WI-9214	12p13	–	Db	(Lopez-Cabrera *et al.*, 1993; Schnittger *et al.*, 1993)
CD94	Type 2 membrane glycoprotein	–	–	12p13	–	Py	(Chang *et al.*, 1995)
GDF3	Growth differentiation factor 3	–	–	12p13	–	Ph	(Caricasole *et al.*, in press.)
GRIN2B	Glutamate receptor	–	–	12p12	–	Sc	(Mandich *et al.*, 1994)
IAPP	Islet amyloid polypeptide	803B2/934H5	–	12p12	–	Sc	(Hoovers *et al.*, 1993; Raeymaekers *et al.*, 1995)
ITPR2	Type 2 inositol 1,4,5-triphosphate receptor	ICI36ECI/798D1 744G11/753F12	D12S2033	12p12	–	Py,Db	(Heighway *et al.*, 1996)
JAW1	Lymphoid marker	753F12/928G2/76-1B8	D12S130E		+	Dd,Py	(Behrens *et al.*, 1994; Behrens *et al.*, 1996)
KRAG	Kirsten ras associated gene	ICI36ECI/798D1 744G11/753F12	–	12p12.1	–	Py	(Heighway *et al.*, 1996)
KRAS2	Signal transduction protein (P21)	761B8/753F12	–	12p12.1	+	Sg,Py	
LDHB	Lactate dehydrogenase isoenzyme B	803B2/957B7 934H5/845D11	–	12p12.1	–	Py	(Raeymakers *et al.*, 1995)
LGP	Liver Glucogen Phosphorylase	754C1	D12S2025		–	Db	
LGS	Liver Glycogen Synthase	803B2/957B7 934H5/845D11	D12S1945		–	Py,Db	(Nuttall *et al.*, 1994)
PTHLH	Parathyroid like hormone	754C1	WI-9193		–	Db	(Raeymakers *et al.*, 1995)
RECQL	DNA helicase Q1	934H5	–		–	Py	(Seki *et al.*, 1994; Larramendy *et al.*, 1997)
SOX5	Sex determining region Y box 5	922D9/y900G1253	–	12p12	+	Py	(Wunderle *et al.*, 1996)
TNFR1	Tumor necrosis factor α receptor	–	–	12p13	–		(Krauter *et al.*, 1995)

[a] – = mapped outside the SROA, + = mapped within the SROA. [b] Db = data obtained from the Whitehead Institute and/or the Human Genome Database. Ph = PCR of a gene specific primer pair to the chromosome 12p hybrid panel. Py = PCR of a gene specific primer pair on the YAC-contig. S = Southern blot analysis using a cDNA probe (Sc) or genomic probe (Sg)

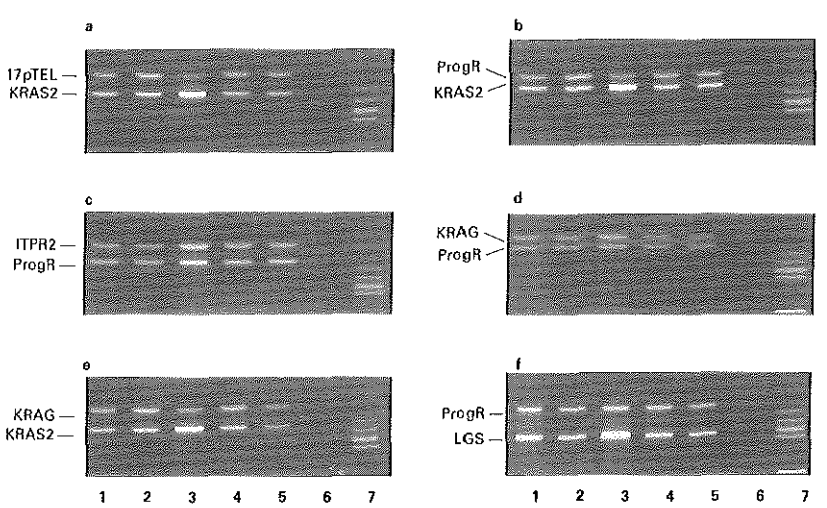

Figure 3 Results of semi-quantitative polymerase chain reactions on tumor T8763 and four control samples. Gels (a) and (b) show amplification in the tumor of *KRAS2 versus* control loci. (c and d) show equivalent levels of *ITPR2* and *KRAG versus* control loci levels, whilst (e) confirms tumor amplification of *KRAS2 versus KRAG*. (f) shows amplification of *LGS versus ProgR* but the degree of amplification seems to be reduced compared to *KRAS2* levels. Tracks: 1, 2, 4, 5 = DNA derived from normal lung tissue; 3 = T8763; 6 = negative control (no target DNA); 7 = ØX174/*Hae*III marker

tumors (see Figure 1b). The results suggest that in both cases a region encoding *KRAS2* but excluding *KRAG/ITPR2* is preferentially amplified. The fact that *LGS* has a similar level in PCR-amplification in tumor T60 as in tumor T8763 is most likely explained by the relatively low percentage of tumor cells containing the smallest amplified region on 12p in the latter sample as demonstrated by FISH with cosmid 235C1.

In addition, the levels of amplification of *PTHLH* and *ITPR2* relative to *KRAS2* were determined. No differences were found between *ITPR2* and *KRAS2* in the tumors T10077, T3035 and T2814, again in agreement with the bicolour-FISH results (Figure 1b). *ITPR2* was not found to be amplified in tumors T60 and T8763. When comparing *KRAS2* with *PTHLH*, four of the tumors (T3035, T2814, T60 and T8763) showed a higher amplification of *KRAS2*, also concordant with the data obtained by bicolour-FISH. Although the bicolour-FISH with YAC#5 and cosmid 34F8 showed differences in signal numbers in tumor T10077 (Figure 1b), no differences in levels of PCR-amplification as compared to *KRAS2* were found with specific primers for STS D12S1688, for which 34F8 is positive. The differences between double-FISH and PCR could be due to the heterogeneity in length and number of the amplicons and the difference in sensitivity of both methods to detect them. No difference in level of PCR-amplification compared to *KRAS2* was found for the STSs D12S1606 and D12S1596 in tumors T10077, T60 and T8763. As the localization of *SOX5* and *JAW1* was identified at a later stage during our investigation, these were not included in the semi-quantitative analysis.

In order to study the expression of the *SOX5* and *KRAS2* genes mapped within the SROA, a semi-quantitative reverse transcription (RT–PCR) was performed. As illustrated in Figure 4, most tumors with amplification of a restricted region of 12p showed a similar level of expression of *KRAS2*. In one tumor a lower level was found. In contrast, a more heterogeneous pattern of expression was found for *SOX5*. In four tumors it was expressed at a lower level than *KRAS2*, and in two at a higher level. In addition, no expression of *SOX5* could be detected in T8763. To exclude loss of this particular genomic fragment in the amplicon, we performed FISH with a YAC containing *SOX5* (y900G1253) simultaneously with YAC#5. Similar numbers of signals of both probes were detected in the tumors with 12p-amplification. All control samples of normal testis parenchyma and parenchyma samples with different amounts of carcinoma *in situ* cells tested showed expression of both *KRAS2* and *SOX5* (not shown). For *JAW1* relatively high expression was found in the tumors containing a 12p-amplification, but also in TGCTs without such an amplicon (not shown). In addition, expression of this gene was also detected in normal testis and placenta.

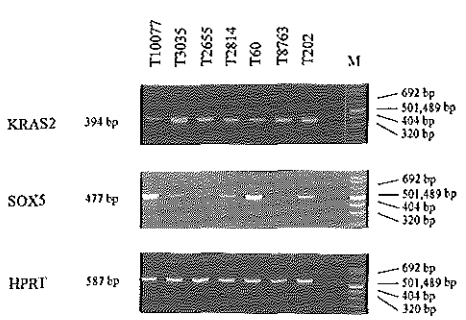

Figure 4 Examples of semi-quantitative reverse transcription polymerase chain reaction analyses of *KRAS2* and *SOX5* as compared to *HPRT* in testicular germ cell tumors of adolescents and adults with amplification of restricted regions on 12p. Note the comparable levels of *KRAS2* expression in all tumors, the variable levels of expression of *SOX5* in the different samples, and the absence of expression of this gene in tumor T8763

Discussion

One of the most important problems in the study of the pathogenesis of TGCTs is the identification of the tumor biologically relevant gene or genes in the over-represented region of 12p. It has been known for more than a decade that the majority of TGCTs contains one or more isochromosomes of the short arm of chromosome 12 (Mukherjee *et al.*, 1991; Rodriquez *et al.*, 1993a; Van Echten *et al.*, 1995; Mostert *et al.*, 1996b). Moreover, it has been demonstrated, that TGCTs without i(12p) also show over-representation of 12p-sequences (Suijkerbuijk *et al.*, 1993; Rodriquez *et al.*, 1993b; Smolarek *et al.*, 1995). Since in those studies a paint for the whole short arm of chromosome 12 was used, or a probe which was not mapped in detail, it was not possible to localize the region involved. The finding of amplification of a restricted region of 12p in a case of metastatic SE (Suijkerbuijk *et al.*, 1994) was a key observation. More recently, we and others reported the presence of similar amplifications in several primary TGCTs (Mostert *et al.*, 1996a; Korn *et al.*, 1996). Apparently gain of 12p-sequences by means of amplification of a smaller region of 12p (instead of i(12p)-formation) is not restricted to metastatic lesions.

We found amplification of a limited region of 12p in eight out of 49 primary TGCTs. In this relatively small and selected sample it occurred in SE as well as NS, suggesting that this type of amplification is not correlated with the histology of the TGCT. Amplification of a restricted region of 12p was preferentially found in TGCTs without i(12p) (six out of 23 cases (26%) and none out of 13 TGCTs with i(12p)). We demonstrate that the borders of the 12p-amplicon vary in different TGCTs, thereby defining the SROA as the region of interest. By assuming that the STSs are equally divided over the YACs 753F12, 922D9 and 957B7, we estimate that the SROA could be as small as 1750 kb. However, when the average distance between markers on chromosome 12 is 248 kb as suggested by Krauter *et al.* (1995) the SROA could be as large as 3000 kb. Although this is still a relatively large fragment, it drastically reduces the crucial region on the short arm of chromosome 12 involved in the pathogenesis of TGCTs.

Identification of this SROA permits the selection of the most likely genes involved in the pathogenesis of TGCTs. For example, cyclin D2 (*CCND2*), mapped to 12p13 (Inaba *et al.*, 1992), was recently reported as the

gene of interest (Sicinski *et al.*, 1996; Houldsworth *et al.*, 1997). We demonstrated by Southern blot analysis using a cDNA probe on all YACs within that region that cyclin D2 maps outside the SROA (not shown). Obviously, cyclin D2 is not likely to be the gene explaining the gain of 12p-sequences in TGCTs. The published findings support this view, because this gene is preferentially expressed in yolk sac tumors and not for example in embryonal carcinoma cell lines (Sicinski *et al.*, 1996), being representative for the stem cells of NS (Andrews *et al.*, 1990). The fact that the presence of the cyclin D2 in NS depends on the histology (Houldsworth *et al.*, 1997) is neutral with respect to the hypothesis that cyclin D2 is the gene of interest. Over-representation of the cyclin D2 gene in most of the TGCTs and derived cell lines (Sicinski *et al.*, 1996) probably merely reflects the over-representation of the complete short arm of chromosome 12 found in virtually all TGCTs (Mukherjee *et al.*, 1991; Rodriquez *et al.*, 1993a,b; Van Echten *et al.*, 1995; Mostert *et al.*, 1996b). A number of other genes previously proposed as candidates we also considered less likely because they map outside the SROA (Table 1). Some of these candidates were plausible, like *LGS*, encoding the rate limiting enzyme in the synthesis of glycogen (Nuttall *et al.*, 1994) and *LDH*B, encoding lactate dehydrogenase type B (Li *et al.*, 1988). Glycogen is found abundantly in carcinoma *in situ* and the majority of the different invasive components (Mostofi *et al.*, 1987). *LDH*B is used as a serum marker for TGCTs, in which its level seems to correlate with the copy numbers of the short arm of chromosome 12 (Taylor *et al.*, 1986; Von Eyben *et al.*, 1988, 1992). *TNFR*1, also located on 12p13 (Fuchs *et al.*, 1992; Krauter *et al.*, 1995) might be a candidate because mouse primordial germ cells, the mouse equivalent of the normal human counterpart of carcinoma *in situ* and SE, show proliferation after TNF exposure (Kawase *et al.*, 1994). However, *LGS*, *LDH*B and *TNFR*1 map outside the SROA.

We reduced the size of the crucial region on 12p to a minimal length of about 1750 kb. Theoretically, between 25–50 genes could be present within such a stretch of DNA. However, *SOX5*, *JAW1* and *KRAS2* are the only genes which were so far mapped within this SROA (Popescu *et al.*, 1985; Wunderle *et al.*, 1996 and this manuscript). The human *SOX5* gene was only very recently described. Studies in the adult mouse showed expression of this gene in haploid round spermatids, where the protein may function as a transcription factor. Human *SOX5* transcripts have been found in adult testis, heart, liver, lung, kidney, spleen and fetal brain. Because we found one TGCT without expression of this gene, *SOX5* does not seem to be the gene of interest. Involvement of *KRAS2* in the pathogenesis of TGCTs has been suggested and questioned by different investigators and its role is largely unclear so far (Dmitrovsky *et al.*, 1990; Moul *et al.*, 1992; Olie *et al.*, 1995). Moreover, it has been reported that activation of *KRAS2* is not sufficient for malignant transformation (Kumar *et al.*, 1990; Rodenhuis, 1992; McCormick, 1994).

*Jaw*1 is expressed in a developmental stage-specific fashion in both mouse B- and T-lymphocyte lineages, with the highest expression levels found in pre-B cells, mature B cells and pre-T cells (Behrens *et al.*, 1994, 1996). No detectable expression was found in non-hematopoietic cell lines. The encoded protein is localized on the cytoplasmic face of the endoplasmatic reticulum (Behrens *et al.*, 1994, 1996). The exact function of this protein is not yet known and the role it might have in cancer pathogenesis is not clear. The *JAW*1 expression as we found in TGCTs might be related to lymphocytic infiltrations, which are most profoundly present in SE (Mostofi *et al.*, 1987), although expression of this gene was also found in NS, which are known to contain significantly less lymphocytic infiltrates (Mostofi *et al.*, 1987).

Gain of 12p-sequences as an initiating event in the pathogenesis of TGCTs seems unlikely, because we reported that polyploidization, resulting in consistent aneuploidy of TGCTs (Oosterhuis *et al.*, 1989; Looijenga *et al.*, 1991), precedes i(12p) formation (Geurts van Kessel *et al.*, 1989) and, therefore, probably also amplification of 12p-sequences. This finding and the fact that amplifications of 12p sequences within one TGCT can be heterogeneous suggests that the increasing copy number is a progression-related phenomenon. This indicates that the pathogenesis of TGCTs is not a single step process. To this day it remains to be elucidated why a number of tumors preferentially amplify a specific region of 12p in contrast to gain of the complete short arm as found in the majority of TGCTs. Proving that *SOX5*, *JAW*1, *KRAS2*, or any other gene mapping within the SROA is the biologically relevant gene for the development of TGCTs, requires more extensive expression analyses at different developmental stages during the pathogenesis of TGCTs, and the development of proper *in vitro* and *in vivo* model systems to test these genes.

In conclusion, we report on the reduction of the region on the short arm of chromosome 12 crucial in the pathogenesis of TGCTs to 1750–3000 kb. This will facilitate the development of assays for the early identification of this chromosomal anomaly and moreover, the amplicon on 12p is a starting point for cloning and characterizing novel genes, which by their mere presence in this region are potential candidate genes.

Materials and methods

Samples

The TGCTs included in this study were collected at the operating theatre in collaboration with Urology- and Pathology-departments in the South-Western part of the Netherlands. After incision of the orchidectomy-specimen, representative samples of the tumor and adjacent parenchyma (when available) were snap frozen in liquid nitrogen, and formaline-fixed and subsequently paraffin embedded for histological examination. The tumors were classified according to the World Health Organization classification for testicular tumors (Mostofi and Price, 1973).

Slide preparation for conventional chromosome banding, FISH and CGH

After surgical removal, representative parts of SE were directly handled to isolate metaphase spreads as described before (Castedo *et al.*, 1989). Briefly, the mitotic cells were, after mechanical dissociation of the tumor, directly harvested in the presence of colcemid (Life Technologies). The cells were subsequently swollen in hypotonic KCl/

EGTA/Hepes solution and fixed with methanol-acetic acid (3:1). Representative parts of NS were enzymatically digested (collagenase, Sigma), and short term cultured in T75 flasks (Costar) under standard conditions, i.e., 37°C, 5% CO_2 in air humidified atmosphere in DMEM/HF12 with 10% heat-inactivated FCS (BRL–GIBCO). Mitotic cells were harvested after 2–4 h of colcemid treatment, again swollen in hypotonic solution and fixed in methanol:acetic acid. For conventional karyotyping, air-dried preparations were banded with pancreatin (Sigma) as reported previously (Mostert *et al.*, 1996b). The chromosome constitution was described according to the International System for Human Cytogenetic Nomenclature 1995 (Mitelman, 1995).

Slides for single- and bicolour-FISH were either prepared from methanol-acetic acid fixed material as reported previously (Mostert *et al.*, 1996b), or from nuclei isolated from frozen material. For the latter method ten frozen tissue sections of 50 μm thickness, of a histologically checked sample, were resuspended in 1 ml PBS by pipetting. The nuclei were directly fixed by adding 70% ethanol (−20°C). After centrifugation for 10 min at 1200 r.p.m., the supernatant was replaced by 5 ml 70% fresh ethanol (−20°C). Finally, fixed suspensions were dropped on slides, air dried and baked for 30 min at 65°C.

For CGH, metaphase spreads were prepared using standard procedures from *in vitro* phytohemagglutinin-stimulated peripheral blood lymphocyte cultures of a healthy male (46,XY), as reported before (Mostert *et al.*, 1996a).

Comparative genomic hybridization

High molecular weight DNAs were isolated from histologically checked samples using standard procedures (proteinase K-sodium dodecyl sulphate treatment followed by phenol-chloroform extraction and ethanol precipitation (Maniatis *et al.*, 1982)). CGH was performed as described (Mostert *et al.*, 1996a) with a few modifications. For each hybridization, 200 ng lissamine-labeled (11-dUTP, Dupont) control male DNA and 200 ng digoxigenin-labeled (11-dUTP, Boehringer Mannheim) tumor DNA and 10 μg COT 1 DNA dissolved in 10 μl standard hybridization mixture were hybridized to normal metaphases and incubated at 37°C for 4 days. The hybridized slides were washed three times with 2× SSC at 37°C followed by 0.1× SSC at 60°C (three washes). Fluorescence detection of the tumor DNA was accomplished using one layer of anti-digoxigenin-FITC (Boehringer Mannheim). The slides were mounted in antifade (Vectashield) containing 4,6-diamidino-2-phenylindole (DAPI, Sigma) for counterstaining of the chromosomes.

The results were evaluated using an epifluorescence microscope (Leica DM) equipped with a cooled CCD camera (Photometrics, Tucson, AZ), a triple band pass beam splitter and emission filters (P-1 filter set, Chroma Technology). Images were saved using a routine built up in SCIL–Image implemented on a Power Macintosh 8100. Image analyses were performed with the use of QUIPS XL software (Vysis Inc. Downers Grove, IL). The green and red fluorescent intensities were measured with the Xwoolz software for a Sun work station. In the first experiment the normalization was carried out as described before (Mostert *et al.*, 1996a), by using the average of the green to red fluorescent intensities for the entire metaphase. Regions that showed variations in centromeric and heterochromatic regions (chromosome 1, 9, 16 and Y) and the immediate telomeric regions were omitted from analysis.

Yeast artificial chromosomes: origin and characterization

YAC#5 was kindly provided by Dr B Gemmill (Denver, USA). The *SOX5* containing YAC ICRFy900G1253 was obtained through the Resource Center in Berlin. YAC ICI36ECI was obtained by Dr J Heighway. All other YACs used are from the CEPH mega-YAC library (contig WC 12.1) and were obtained through the Leiden YAC Screening Centre. YAC clones were grown and DNA was isolated according to standard protocols (Mostert *et al.*, 1996b). Proper localization of the YACs was tested by FISH on normal human lymphocytes. The length of the YACs was determined by pulsed field gel electrophoresis.

Inter-Alu PCR on YAC#5 and other YACs from the contig was performed using primers ALUI and ALUIV (Cotter *et al.*, 1991).

STS and cosmids

Cosmids mapping within the contig were isolated from a chromosome 12 specific library by hybridization of spotted filters with amplified fragments generated by PCR using STS-specific primer products isolated from normal human placenta. The STS-specific primer sequences were obtained from the Whitehead Institute for Biomedical Research database, and additional information (localization, polymorphism, PCR conditions, etc.) was obtained from the Genome Database. The reactions were performed on 250–500 ng DNA, in 50 μl reaction volume (containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP and 0.2 units of Taq polymerase), as well as the different STS-specific primers. After initial heating to 94°C for 5 min, followed by 30–35 cycles of DNA denaturation (1 min at 94°C), annealing (1 min at 55°C, 58°C or 60°C depending on the primer set used) and extension (1 min at 72°C), final extension was performed at 72°C for 10 min. Five μl of the volume was analysed by electrophoresis on a 3% agarose gel (50% regular and 50% NuSieve GTG agarose) and ethidium bromide staining. The different PCR products were isolated from gel using the Quiaquick Gel Extraction Kit (Quiagen). Subsequently, 100 ng was radioactively labeled using random primed labeling and hybridized to the chromosome 12-specific cosmid-library. Sonicated placental DNA was used to reduce background staining. In addition, CA repeat containing STS fragments were prehybridized with other CA repeat-containing probes for 1 h in 5× SSC. For STS fragments smaller than 200 bp, end-labeled oligo's were used as probe. Positive cosmid clones were checked for their specificity for the relevant STS by PCR using 50 ng cosmid DNA. In addition, the cosmids were tested for their suitability for FISH on normal lymphocyte-preparations.

FISH: labeling of the probes and hybridization

The single and bicolour-FISH experiments were performed as described earlier (Mostert *et al.*, 1996b) using YAC#5 and either one of the other YACs or cosmids as probe. YAC#5 was purified by pulsed field gel electrophoresis, amplified and labeled with digoxigenin-11-dUTP (GIBCO–BRL) as reported before. The other probes were labeled using a standard nick-translation kit (GIBCO–BRL) with biotin-11-dUTP. One hundred ng of both labeled probes and 0.5 μg COT 1 DNA (GIBCO–BRL) were dissolved in 10 μl standard hybridization mixture containing 50% formamide/2× SSC/10% dextran-sulphate. The slides were prepared from methanol-acetic acid fixed sediment from cases T2814, T8763 and T202 and from ethanol fixed suspensions containing nuclei from frozen tissue of cases T10077, T3035, T2207, T2655 and T60. Before hybridization, the slides were pretreated as follows: the methanol/acetic acid fixed slides were rinsed in water and incubated in 50% HAc/H_2O and 50% HAc/MeOH for 4 min respectively and airdried. The slides were dehydrated in 70% ethanol (0°C) and subsequently in 80%, 90% and 100% at room temperature for 3 min, respec-

tively. After air-drying, the slides were treated with RNase (0.1 mg/ml) (SIGMA) in 2× SSC for 30 min at room temperature and rinsed in 2× SSC. The ethanol fixed slides were incubated in 0.1 mg/ml pepsine in 0.01% HCL (SIGMA) for 20 min at 37°C. After rinsing in water and PBS, five times each, the nuclei were post-fixed with 1% formaldehyde in PBS for 10 min at 4°C. After another five rinses with PBS the nuclei were dehydrated in 70%, 80%, 90% and 100% ethanol, respectively. After *in situ* hybridization for 48 h at 37°C, the biotin labeled probes were visualized by a new detection method with biotinyl-tyramide (Raap *et al.*, 1995) with a few modifications. Briefly, the slides were incubated with ABC (DAKO) streptavidin-peroxide for 30 min at room temperature. Following washes with PBS/0.1% Tween, the biotinyl-tyramide solution (NEP-116 kit of Dupont) 1:200–1:400, 0.01% H_2O_2 in PBS was incubated for 10 min at room temperature. After enzymatic reaction, probe YAC#5 was visualized with fluorescein isothiocyanate (FITC) conjugated sheep-α-digoxigenin (Boehringer) and the biotin labeled probe with avidin-CY3 (Jacksons Immunoresearch Laboratories).

For every tumor, 30 nuclei with amplification of YAC#5 were analysed by three different investigators. In each nucleus, the number of hybridization signals of YAC#5 (green) was compared to the number of signals of the other probe (detected in red).

Mapping of candidate genes and expression analysis

LGS-specific primers were generated within the 3'UTR (positions 2349–2368 and 2610–2631, GENBank accession number S70004), with the following sequences: LGS1: 5'-AGTTAGGCATGAGGAGGAGC-3', and LGS2: 5'-AATCATGGTTCTGATGCATGTG-3'. The primers used for *SOX5* were SOX5f2 (5'-CACTGCTGGTGTTGTG-TAC-3') and 2E2reva (Wunderle *et al.*, 1996), and for *KRAS2* KR1 (5'-TGATAGTGTATTAACCTTATG-3') and KR2 (5'-TTTATCTGTATCAAAGAATG-3') (both in exon 1). The primers for *JAW1* (5'-CAGCAGGACATCC-TAATATATGG-3' and 5'-CCCATTGTATTTTAGTTG-GGG-3') were the STS primers for D12S1350E. The PCR experiments were performed as described in the Results section.

RT–PCR was performed on total RNA isolated from approximately five tissue sections of 50 μm thickness of the snap-frozen samples, using RNA STAT-60 (TEL-TEST). Of every sample, two 5 μm sections (the first and the last in the series) were stained with hematoxylin and eosin for microscopic analysis of the histological composition. After treatment of the RNA with RNase-free DNase (Boehringer), phenol/chloroform extraction and precipitation, 5 μg of total RNA was reverse transcribed at 37°C for 2 h in a total volume of 40 μl containing 1 mM each dNTP (Pharmacia), 1 mM dithiothreitol, 1.2 μg random hexamer primers (pd[N]6) (Pharmacia), 1.2 μg oligo d(T) primer d[T]12-18 (Pharmacia), 4.5 U of RNasin (Pharmacia), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 1 μl of Superscript RNAse H⁻ RT (BRL; 200 U/μl). Absence of contaminating DNA was checked by PCR without prior reverse transcription. Integrity of the cDNA was tested via amplification of Hypoxanthine Phospho-Ribosyl-Transferase (*HPRT*) transcripts (28 cycles), with primers 244 and 246 as published before (Gibbs *et al.*, 1989) (5'-AATTATGGACAGGACT-GAACGTC-3' and 5'-GGCGATGTCAATAGGACTCCA-GATG-3', respectively), generating a fragment of 587 bp. Amplification was performed using 1.5 μl of the same reverse transcription reaction mix in a total volume of 50 μl containing 1× Taq DNA polymerase buffer with 1.5 mM $MgCl_2$, 25 pmol of each primer, 0.2 mM each dNTP and 1 U of Taq DNA polymerase (Promega). After an initial denaturation of 4 min at 94°C, every amplification cycle consisted (in total 32) of 1 min at 95°C, 1 min at 64°C and 1.5 min at 72°C. PCR products were visualized on 3% (50% regular and 50% NuSieve GTG (FMC)) agarose gels stained with ethidium bromide. This was also used as reference for the level of expression of *SOX5* and *KRAS2*. The primers used for *SOX5* (spanning an intron) were SOX5fore and 2E2reva (Wunderle *et al.*, 1996), generating a fragment of 477 bp. Primers (spanning two introns) used for *KRAS* were KR11 in exon1 (5'-GAGTGCCTTGACGATACAGCT-3') and KR12 in exon 3 (5'-CTTTGCTGATGTTTCAATAA-AAGG-3'), generating a fragment of 394 bp. Amplification of *SOX5* and *KRAS2* transcripts was done using an initial denaturation of 5 min at 94°C, and subsequently 35 cycles consisted of 45 s 94°C, 30 s 60°C and 30 s 72°C, and finally 10 min at 72°C for *SOX5* and after initial denaturation 35 cycles consisted of 1 min 94°C, 1 min 58°C and 1 min 72°C, and finally 10 min at 72°C, for *KRAS2*.

Semi-quantitative PCR

The semi-quantitative PCR was performed as described before (Heighway *et al.*, 1996). Besides the seven TGCTs with the amplification of 12p sequences, seven TGCTs without such amplifications were included as controls. The cases were studied under code not knowing the origin of the samples. The progesterone receptor (*ProgR*), which maps to 11q23 and the *17pTEL* STS, were included in every experiment as references (Bettincher *et al.*, 1996; White *et al.*, 1996). The following target genes were studied: *LGS*, *KRAS2*, *KRAG* (primers 021/022) and *ITPR2*. Briefly, for each PCR 0.5 μg tumor DNA was amplified in a 100 μl reaction with 0.5 μg of each primer (two primer pairs) in standard reaction mix over 30 cycles of 1 min at 55–60°C, 1 min at 74°C, 1 min at 94°C and final steps of 1 min at 55–60°C and 10 min 74°C. After gel electrophoresis (2% agarose), relative band intensities of PCR products (test *versus* control) were compared visually, with reference to band intensities derived from normal genomic DNA controls.

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Chapter 7

Role of restricted 12p-amplification and *RAS* mutation in the development and clinical behaviour of human testicular germ cell tumors of adolescents and adults

submitted

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Abstract

Human testicular germ cell tumors of adolescents and young adults (TGCT), histologically seminomas and nonseminomas, are characterized by 12p-overrepresentation, mostly due to isochromosomes of 12p, of which the biological and clinical significance is still unclear. A limited number of TGCT has been identified with an additional high level amplification of a restricted region of 12p, including the *K-RAS* proto-oncogene. Here we show that the incidence of these restricted 12p-amplifications in primary TGCT is about 8%, affecting both seminomas and nonseminomas. Formation of i(12p) and restricted 12p-amplification is mutually exclusive in a single cell. The shortest region of overlap of amplification was not reduced compared to previous reports. It appears that the borders of the amplicons cluster in short regions. While most seminomas showed the amplification in all tumor cells (homogeneous pattern), only a minority of the tumor cells from nonseminomas contained the amplicon (heterogeneous pattern). The amplicon was never found in the adjacent carcinoma *in situ* cells. Seminomas with the restricted 12p-amplification virtually lacked apoptosis, and the tumor cells showed prolonged *in vitro* survival, like seminoma cells with a mutated *RAS* gene. No differences in proliferation index were found between these different groups of seminomas. Seminoma patients with a homogeneous restricted 12p-amplification presented at a significantly younger age than those lacking it. The presence of a restricted 12p-amplification/*RAS* mutation did not predict the stage of the disease at clinical presentation and the treatment response of primary seminomas. In 55 primary and metastatic tumors from 44 different patients who failed cisplatinum-based chemotherapy the restricted 12p-amplification and *RAS*-mutations had the same incidence as in the consecutive series of responding patients.

These data support the model that gain of 12p is progression-related in the development of TGCT: it allows tumor cells, in particular those showing characteristics of early germ cells, i.e. the seminoma cells, to survive outside their specific micro-environment. Probably overexpression of certain genes on 12p inhibit apoptosis in these tumor cells. However, the copy numbers of the restricted amplification of 12p and *K-RAS* mutations do not predict response to therapy and survival of the patients.

Introduction

Malignant transformation is a complex, multistep process, in which inactivation of tumor suppressor genes and activation of proto-oncogenes play an important role (Vogelstein & Kinzler, 1993). Although involvement of several genes has been suggested in the development of testicular germ cell tumors of adolescents and young adults (TGCT), histologically seminoma and nonseminomas (Mostofi *et al.*, 1987), evidence is lacking.

All TGCT originate from carcinoma *in situ* (CIS) (Skakkebæk, 1972), composed of tumor cells that phenotypically resemble primordial germ cells (Jørgensen *et al.*, 1995). CIS is frequently found in the parenchyma adjacent to an invasive TGCT. These cells are located on the inner side of the basal membrane of the seminiferous tubules, in close association with Sertoli cells (Gondos, 1993). Initially they expand in the seminiferous tubules, eventually, via the micro-invasive stage, the tumor cells form overt invasive tumors. The mechanisms involved in this progression are still unclear. Obviously tumor cells are selected capable to survive and grow outside the specific micro-environment of the seminiferous tubule.

The only consistent chromosomal anomaly in TGCT is gain of the short arm of chromosome 12, mostly due to isochromosomes of 12p (Atkin & Baker, 1983; Sandberg *et al.* 1996, for review). *K-RAS* has been proposed as the relevant gene (Dmitrovsky *et al.*, 1990; Houldsworth *et al.*, 1997; Murty & Chaganti, 1998). The copy number of 12p reportedly predicts prognosis (Bosl & Chaganti, 1994; Bosl *et al.*, 1989; Malek *et al.*, 1997). The *RAS* protein (p21) is involved in signal transduction by switching between an inactive (GDP-bound) and active (GTP-bound) state. Mutations leading to constitutive activation have been found in many types of cancers. For example, mutated *RAS* correlates with poor prognosis in childhood acute lymphocytic leukemia (Lübbert *et al.*, 1990), and non-small cell lung cancer (Slebos *et al.*, 1990; Mitsudomi *et al.*, 1991). Recently, activation of *RAS* has been shown to be involved in tumor-maintenance (Chin *et al.*, 1999; Hahn *et al.*, 1999), and in inducing anchorage independent growth, due to inhibition of apoptosis (Arends *et al.*, 1993; Frisch & Francis, 1994). Indeed, a correlation between activated *RAS* and metastatic capacity has been reported (Ananthaswamy *et al.*, 1989; Takiguchi *et al.*, 1992). Besides activation by means of mutations, *RAS* can also be involved in malignant transformation due to increased copy numbers of the wild type gene, resulting in overexpression of wild type mRNA, and accumulation of the wild type protein (Coleman *et al.*, 1994; Fujita *et al.*, 1987; McKay *et al.*, 1986; Pulciani *et al.*, 1985; Radinsky *et al.*, 1987; Zhang *et al.*, 1997).

Seminomas are highly sensitive to irradiation and cisplatinum-based chemotherapy. The vast majority of patients with metastatic nonseminomas is cured by cisplatinum-based combination chemotherapy (Bokemeyer & Schmoll, 1993, for review). In spite of the application of clinical and histological prognostic factors to predict treatment response of TGCT, the clinical course of these tumors can still not be predicted for individual patients. Additional prognostic markers are therefore needed. Interestingly, activated *RAS* genes increase the intrinsic resistance to radiation and cisplatinum therapy (Holford *et al.*, 1998; Isonishi *et al.*, 1991; Kinashi *et al.*, 1998;

Sklar, 1988a; Sklar, 1988b). It is unclear whether amplification of wild type *RAS* has the same effect.

A small percentage of TGCT has *RAS* mutations (Ganguly *et al.*, 1990; Moul *et al.*, 1992; Olie *et al.*, 1995a). The clinical relevance of the mutations was not studied. We showed that seminomas with a mutated *RAS* gene have survival advantage *in vitro*, and have reduced apoptosis in the primary tumor (Olie *et al.*, 1996; Olie *et al.*, 1995b). Noteworthy, *in vitro* survival of tumor cells correlates with poor prognosis in patients with adult acute myeloid leukemia (Löwenberg *et al.*, 1993), and in metastatic TGCT (Otto *et al.*, 1997). Recently, a number of invasive TGCT with amplification of a restricted region of 12p have been identified (Korn *et al.*, 1996; Mostert *et al.*, 1998; Mostert *et al.*, 1996; Suijkerbuijk *et al.*, 1994). We showed that the shortest region of overlap of amplification (SROA) is about 1.7 Mbases, containing three known genes, i.e., *SOX5*, *JAW1*, and *K-RAS* (Mostert *et al.*, 1998). It is unknown so far whether amplification of wild type *K-RAS* in these tumors has the same effects as *RAS* mutations. Finally, the clinical relevance of a restricted 12p-amplification has not been conclusively investigated in TGCT.

The goal of this study is to further investigate the biological and clinical significance of gain of 12p-sequences in TGCT. The incidence of restricted 12p-amplification was studied in a consecutive series of 76 untreated primary TGCT. The newly identified six tumors, as well as previously found nine cases were studied for the distribution of the restricted 12p-amplification within the tumor (homogeneous or heterogeneous pattern), the borders of the SROA, the presence of i(12p), the proliferative activity, the presence of apoptosis and capacity of the tumor cells to survive *in vitro*. In addition, corresponding CIS and micro-invasive seminoma were tested for the presence of the restricted 12p-amplification. The clinical importance of the restricted 12p-amplification and *RAS* mutation was further studied in 44 patients who failed cisplatinum-based chemotherapy.

Material and methods

Samples

The freshly obtained tumor samples included in this study were collected in close collaboration with urologists and pathologists in the South-Western part of the Netherlands. All tumors were obtained prior to chemotherapy and/or irradiation. Directly after surgical removal, representative parts of the tumor and adjacent normal tissue (when available) were snap frozen and other pieces were fixed overnight in 10% buffered formalin and embedded in paraffin. The size of the testis and the tumor was measured in three dimensions. The tumors were diagnosed according to the World Health Organisation (WHO) classification for testicular tumors (Mostofi & Sesterhenn, 1998). Nonseminomas containing both a seminoma and a nonseminoma were classified as combined tumors, according to the British classification (Pugh, 1976), instead of as nonseminomas according to the WHO classification system. Identification of CIS, seminoma and embryonal carcinoma was aided by a direct enzyme-histochemical detection of alkaline phosphatase activity on representative frozen tissue sections, as reported before (Mosselman *et al.*, 1996). The consecutive series tested for the presence of a restricted 12p-amplification (see below) consisted of 46 seminomas, 23 nonseminomas (14 embryonal carcinomas/yolk sac tumor/teratoma; 3 teratomas; 2 embryonal carcinoma/yolk sac tumor; 2 yolk sac tumors, 1 teratoma/yolk sac tumor; 1 embryonal carcinoma) and seven combined tumors (3 embryonal carcinoma/seminoma; 3 embryonal carcinoma/yolk sac/seminoma; 1 teratoma/yolk sac tumor/seminoma). The newly identified cases with a restricted 12p-amplification and the previously found cases (Mostert *et al.*, 1998 and unpublished observations) were studied for the borders of the amplicon (see below), the distribution within the tumor (see below), presence of i(12p) by karyotyping (when available), proliferation index (see below), apoptosis and *in vitro* survival, as described before (see below, and Olie *et al.*, 1996; Olie *et al.*, 1995b). In addition, the formerly identified seminomas with a mutated *RAS* gene (Olie *et al.*, 1995a), were included in this analysis.

The analysis of the possible clinical impact of the restricted 12p-amplification/*K-RAS* mutation was studied on a series of patients who failed cisplatinum based chemotherapy. These cases were collected in collaboration with the Departments of Hematology and Oncology, University of Tübingen (Germany), and Internal Medicine, Netherlands Cancer Institute, Amsterdam (The Netherlands). In total, 44 patients were included, of whom 22 primary TGCT and 33 metastases were studied (of 11 patients both the primary and metastases was available).

Immunohistochemical detection of Ki-67

Sections were cut from one representative paraffin block per tumor, which was mounted on APES (3-aminopropyl-triethoxysilane)-coated slides and dried at 50°C overnight. Subsequently, the sections were heated to 120°C in sodiumcitrate solution (0.01 M pH 8.0) using an autoclave (Shin *et al.*, 1991). Endogenous peroxidase reactivity was blocked with

H_2O_2/methanol. The Ki-67 antigen was demonstrated using the polyclonal antibody A 0047 (Dako), diluted 1:100. Incubation was done for 1 hour at room temperature following blocking of non-specific binding sites with 5% BSA. After extensive washing, biotinylated swine-anti-rabbit (1:200) (Dako) was used as second step, which was detected using the horseradish-labeled streptavidin-biotin complex (Dako), diluted 1:100. Peroxidase was visualized with diaminobenzidine, after which the sections were counterstained lightly with Mayer's hemalum. Of each tumor three times 50 tumor cells were independently counted by two observers in one tissue section. The results were statistically analyzed using the Student's t test. Positive and negative (excluding the first antibody) controls were included in each experiment.

Metaphase preparations

After surgical removal, representative parts of nonseminomas were enzymatically digested (collagenase, Sigma), and cultured in T75 flasks (Costar) for several days under standard conditions, i.e. 37°C in a humidified atmosphere with 5% CO_2 in air in DMEM/HF12 culture medium with 10% heat-inactivated FCS (BRL-GIBCO) as described before (Castedo *et al.*, 1989a). Mitotic cells were harvested after 2-4 hours of colcemid treatment, swollen in hypotonic solution, and fixed in methanol/acetic acid (3:1). Representative parts of seminoma were directly processed to isolate metaphase spreads as described before (Castedo *et al.*, 1989b). Briefly, the mitotic cells were, after mechanical dissociation of the tumor, directly harvested in the presence of colcemid. The cells were subsequently swollen in hypotonic KCl/EGTA/Hepes solution and fixed with methanol:acetic acid (3:1). For conventional G-band karyotyping, air-dried preparations were digested with pancreatin. The chromosome constitution was described according to the International System for Human Cytogenetic Nomenclature 1995 (Mitelman, 1995).

Restricted 12p-amplification

The fluorescence *in situ* hybridisation (FISH) experiments on the methanol/acetic acid fixed nuclei suspensions on the consecutive series were performed as described earlier (Mostert *et al.*, 1998) using YAC#5 (mapped to 12p11.2-12.1, kindly provided by Dr. B. Gemmill, Denver, USA). YAC#5 is known to map to the SROA as determined in our earlier study (Mostert *et al.*, 1998). To make this probe suitable for the FISH approach, it was purified by pulsed field gel electrophoresis, amplified and labeled with digoxigenin-11-dUTP (GIBCO-BRL). It was visualised with fluorescein isothiocyanate (FITC) conjugated sheep-α-digoxigenin (Boehringer) as reported before. The presence of a 12p-amplification was defined as reported before (Mostert *et al.*, 1998), being around 15-30 signals per interphase nucleus.

TGCT identified with a restricted 12p-amplification were studied in more detail. Of these cases, frozen tissue sections of 16 μm thickness containing tumor, micro-invasive seminoma, and CIS (when available), were cut and air-dried overnight at 37°C on microscope slides treated with tissue adhesive. In addition, one parallel section (4 μm thickness) was stained with hematoxylin and eosin, and the other for alkaline phosphatase reactivity, (see above) for histological

examination. The slides for the FISH were submerged in 70% ethanol (-20°C) for one hour and dehydrated in an increasing ethanol series (80%, 90%, 100%, 2 minutes each) and air-dried. Subsequently, the tissue sections underwent protein digestion (0,0005% pepsin (Sigma, St. Louis, USA) in 0,01 M HCl/PBS, 1 minute at 37°C) followed by a wash step (PBS, 5 minutes) and dehydration. Hybridisation was performed as described for the methanol/acidic acid fixed nuclei (Mostert *et al.*, 1998). YAC#5 was used as a control probe in combination with another probe (test probe) (see Figure 1). In particular, probes positive for STSs D12S1945, D12S1688, AFM267yc9 (on the distal side) and D12S1350E, *KRAS2*, D12S1313, and D12S1411 (on the proximal border) were used. YAC#5 was labeled as described above with digoxigenin-11-dUTP (GIBCO-BRL) and visualised with fluorescein isothiocyanate (FITC) conjugated sheep-α-digoxigenin (Boehringer). The test probes were labeled using a nick-translation kit (GIBCO BRL) with biotin-11-dUTP and visualised using avidine-CY3 (Jacksons immunoresearch Laboratories). A restricted 12p-amplification was defined as the presence of nuclei with at least 15 hybridisation signals. The pattern was identified as heterogeneous when such nuclei (possibly in groups) are scattered throughout the tissue section: both regions with and without restricted 12p-amplification are present. In contrast, the pattern is defined as homogeneous when all regions showed nuclei with restricted 12p-amplification. Because of tissue cutting artifacts, this does not exclude the presence of single nuclei without such an amplification. The criteria used to determine the borders of the breakpoints were similar as used in our former study (Mostert *et al.*, 1998). Briefly, the probe was scored at part of the amplicon when paired hybridisation signals with the control probe (YAC#5) was observed, and scored as outside the amplicon when less hybridisation signals were found compared to the control probe. Higher copy numbers of the test probes (see above) compared to the control probes have not been found so far.

The paraffin-embedded tissue sections of the treatment-resistant TGCT (4 µm thickness) were preincubated overnight at 50°C, and subsequently baked for 10 min. at 80°C. The sections were deparaffined using xylene, washed in 100% methanol four times at room temperature and air-dried. Sodium-thiocyanate (1 M) pretreatment was done for 10 minutes at 80°C, after which the slides were thoroughly rinsed in water. Digestion was done using 0.1% pronase E (Sigma) in PBS at 37°C for 30-40 minutes, depending on the tissue under investigation. After rinsing in PBS at 4°C, dehydration was done using a series of increasing ethanol concentrations (70, 80, 90, 100%). The hybridisation, washing and detection procedures were performed as described for the methanol/acetic acid fixed nuclei (see above). A restricted 12p-amplification was defined as the presence of at least 10 hybridisation signals. Because of the thickness of the tissue sections used, the tumors were not scored for the presence of a homogeneous or heterogeneous pattern.

Visualization was done with a Zeiss Axioskop epifluorescence microscope (Carl Zeiss Jena, Germany) with a Pinkel filter in combination with a triple band-pass filter, which enabled the observation of FITC, CY3 and DAPI in one view.

Comparative Genomic Hybridisation (CGH)

For CGH, metaphase spreads were prepared using standard procedures from *in vitro* phytohemagglutinin-stimulated peripheral blood lymphocyte cultures of a healthy male as reported previously (Rosenberg *et al.*, 1998a). High molecular weight DNA was isolated from the snap frozen tissue samples (test DNA) and from peripheral blood of a normal male (reference DNA) using standard procedures (Maniatis *et al.*, 1982). The CGH procedure and analysis was performed as described before (Rosenberg *et al.*, 1998b). Both the control male DNA and the tumor DNA were directly labeled by nicktranslation with lissamine and FITC respectively. The data were analysed using Quips XL software from Vysis (Downers Grove, IL). Normalization was carried out using the average of the green to red fluorescent intensities for the entire metaphase. At least ten metaphases were studied for each case. Losses of DNA sequences were defined as chromosomal regions where the average green-to-red ratio and its 95% confidence intervals are below 0.9 while gains are above 1.1 (Rosenberg *et al.*, 1999). The heterochromatic blocks of chromosome 1, 9, 16 and Y, and the immediate telomeric regions were excluded from the analysis because these regions present variable results in experiments with normal control DNAs.

Spectral karyotyping (SKY)

SKY was performed on a single seminoma with a restricted 12p-amplification, demonstrated both by CGH and FISH, using a SKY-kit and analysis system (Applied Spectral Imaging) with a slightly modified procedure as described by the supplier. In particular, proteinase K digestion was performed in 1 M Tris-HCL, pH 7.5, and after denaturation of the probe, the mix was kept on ice. Washing during the detection procedure was performed in 55% formamide and 39°C in stead of 45°C. In addition, 0.05% instead of 0.1% tween was used in 4 times SSC, and the last washing step was performed at room temperature.

RAS gene mutation detection

Mutations in codon 12 or 13 of the *N-* and *K-RAS* genes were analyzed by direct sequencing (Amplicycle, Amersham), using primer NA (5'-GACTGAGTACAAACTGGTGG-3')/NB (5'-CTCTATGGTGGGATCATATT-3') and KA (5'-GACTGAATATAAACTTGTGG-3')/KB (5'-CTATTGTTGGATCATATTCC-3'), respectively, on DNA isolated from snap frozen seminomas tested for *in vitro* survival and presence of apoptosis. DNA was isolated as decribed above. Only histological areas containing more than 70% tumor cells were used. In addition, the presence of *K-RAS* codon 12 mutations was investigated in the series of paraffin-embedded tumors of the non-responding patients as follows: two 15 μm thickness sections from each sample were first deparaffinized with xylene and then dehydrated with absolute ethanol. A parallel section (4 μm thickness) was stained with hematoxylin and eosin to confirm the presence of tumor, and check for histology. Only sections with a major tumor component were used for DNA isolation. DNA was eluted in 30 ul of water by heating the air-dried tissues at 95°C for 5 minutes. Typically 50-100 ng of DNA was used for each PCR reaction. Each sample was first

tested with HLA-dQ primers (Benhattar *et al.*, 1993) to assess the quality of the eluted DNA. Only samples showing proper amplification were subsequently used. The PCR reactions to detect mutations in codon 12 of *K-RAS* were performed essentially as described before (Kahn *et al.*, 1991) with the following modifications. The *K-RAS* 5' primer used in the two rounds of amplifications carried a biotin label at the 5' end. After the first *Mva* I digestion, the PCR products were affinity purified on streptavidin coated paramagnetic beads (Dynal) and the bound PCR products were re-digested on the beads in 50 ul of *Mva*I buffer containing 10 U of enzyme for 3 hours at 37°C. Subsequently the beads were affinity purified and washed on the magnet to remove the buffer and the unbound fraction. Finally, single stranded DNA was eluted from the bound fraction by boiling the beads with 10 ul of water. One half of each fraction was used in the second amplification step. Samples positive for mutations in codon 12 were further characterized by cycle sequencing the single stranded DNA obtained by affinity purification of the PCR products retained on the beads after the final *Mva*I digestion. The procedure was controlled at every stage with DNA samples obtained from archival paraffin embedded primary TGCT with and without codon 12 mutations (Olie *et al.*, 1995a).

Detection of DNA laddering

High molecular weight DNA was isolated from snap frozen histologically checked samples (see above) using standard procedures (see above). DNA laddering was visualised using ethidium bromide staining after electrophoresis of 1 ug as described previously (Olie *et al.*, 1996).

Results

Restricted 12p-amplication and i(12p) in primary invasive TGCT

The incidence of a restricted 12p-amplification in primary untreated TGCT was determined by analysis of methanol/acetic fixed nuclei suspensions of 76 surgically removed, consecutively collected TGCT, including 46 seminomas, 23 nonseminomas and 7 combined tumors (see Table 1 and **Materials and Methods** for histology). Double fluorescent FISH showed that all tumor nuclei had more than four copies of 12p, and at least three chromosome 12-centromeres (not shown). Six tumors (7.9%), i.e., four seminomas (8.7%), one nonseminoma (4.3%) and one combined tumor (14.2%) showed the restricted 12p-amplification (see Table 1). So far, in total 15 primary TGCT with such a restricted 12p-amplification are available (the six identified in this consecutive study, and nine previously found by us, of which six have been included in our previous study (Mostert *et al.*, 1998): nine seminomas, four nonseminomas and two combined tumors. Karyotyping (see Table 1) revealed the presence of i(12p) in two out of the six successfully analyzed seminomas with a restricted 12p-amplification. However, the presence of an i(12p) could not be confirmed by FISH on matched metaphase spreads, although the amplification was found (not shown). Three of the four karyotyped restricted 12p-amplification positive nonseminomas lacked i(12p), as well as the only karyotyped combined tumor.

Table 1.

Results of detection of the restricted 12p-amplification using methanol/acetic acid fixed nuclei suspensions of a consecutive series of primary seminomas (SE), nonseminomas (NS) and combined tumors (CT) as detected by double fluorescent in situ hybridisation (FISH). The total number of cases with a restricted 12p-amplification identified so far are indicated between brackets. The results of karyotyping and in situ hybridisation on matched frozen tissue sections are also shown (NA = not available; +12p ampl.= with restricted 12p amplification)

	Consecutive series		i(12p) +		Heterogeneity
	tested	+ 12p ampl.	karyotyping	ISH	
SE	46	4 (9)	2/6	0/6	3/9
NS	23	1 (4)	0/3	NA	4/4
CT	7	1 (2)	0/1	NA	2/2
Total:	76	6 (15)			

The other nonseminomas with a restricted 12p-amplification had i(12p) in some of the studied metaphases. Because of inappropriate quality of this material, no FISH could be performed on these cases. In the informative cases, it was found that the additional 12p-sequences visualized by FISH were predominantly (five out the nine cases) present as tandemly organized units as add(12)(p11), add(12)(p12), or add(12)(p13), although also other sites were found to contain 12p-specific sequences, including parts of chromosomes 6, 8, and 11 (not shown).

Breakpoints involved in the restricted 12p-amplification

In addition to our earlier report consisting of six cases (Mostert *et al.*, 1998) (indicated by an asterisk in Figure 1), FISH was applied on the newly identified TGCT (n=9) with a restricted 12p-amplification.

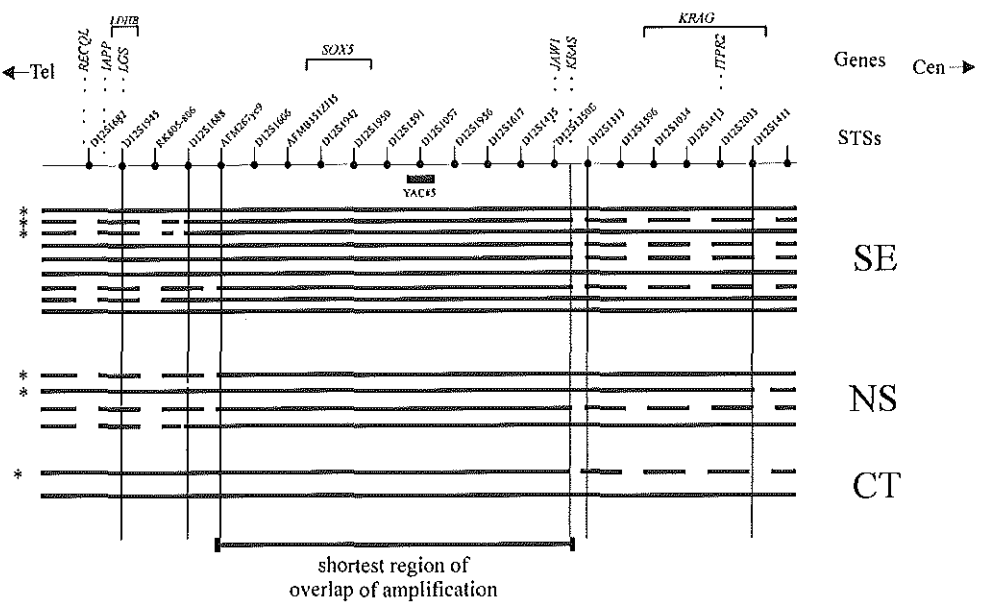


***Fig.1**: Schematic representation of the physical map of the 12p11.2-p12.1 region. Genes, STSs and the position the YAC#5 probe are indicated. The results of the double fluorescent in situ hybridisation experiments performed on frozen tissue sections to determine the shortest region of overlap of amplification are shown. The continuous bold lines indicate the regions included in the restricted 12p-amplification, and the dotted lines indicate the regions outside the amplification. Probes specific for STSs D12S1945, D12S1688, AFM267yc9, K-RAS, D12S1313, and D12S1411 are used for these analyses. The results are shown per histological subgroup (seminomas (SE), nonseminomas (NS), and combined tumors(CT)). The tumors studied in our previous analysis (Mostert et al., 1998), are indicated by an asterisk. Note that three breakpoints map between AFM267yc9 and D12S1688, and seven between AFM267yc9 and D12S1945 at the distal side, and six between K-RAS and D12S1313 at the proximal side. The shortest region of amplification encompasses the genomic fragment between AFM267yc9 and K-RAS.*

Because this analysis was done on frozen tissue sections instead of methanol/acetic acid fixed nuclei suspension, as performed in our earlier study, all cases could be analyzed, including the two previously identified non-informative cases (Mostert *et al.*, 1998). Again YAC#5 was used in combination with probes specific for the more proximal and distal regions of the contig (see Figure 1). In accordance with our previous findings, the amplified region always includes the genomic fragment between STS AFM267yc9 at the distal end, and *K-RAS* at the proximal end. Therefore, *K-RAS*, *JAW1*, and *SOX5* are consistently amplified in all TGCT with a restricted 12p-amplification, irrespective of histology. In addition to this finding, the borders of the amplicon appeared to cluster in narrow regions: 40% between *K-RAS* and STS D12S1313 at the distal side (44.4% for seminoma and 25% for nonseminoma), and 20% between AFM267yc9 and D12S1688 at the proximal side (11% for seminoma and 50% for nonseminoma). In addition, 47% of the breakpoints map between AFM267yc9 and D12S1945 (44% for seminoma and 75% for nonseminoma) (see Figure 1).

Intratumor 12p-amplification and tumor heterogeneity

FISH results on the suspensions of nuclei already indicated that 12p-amplification can be heterogeneously distributed in one tumor (not shown). This was verified by FISH on frozen tissue sections. Six out of the nine seminomas showed the amplification homogeneously throughout the tumor (see Table 1). The other three cases showed regions with and without amplification, of which two contained i(12p) by karyotyping. All nonseminomas and combined tumors showed a heterogeneous pattern. In two cases the amplification was present in a subpopulation of cells of the yolk sac tumor component, and in one in a subpopulation of both the embryonal carcinoma and yolk sac tumor component. One of the combined tumors with the restricted 12p-amplification was a mixture of seminoma and embryonal carcinoma. The amplification was found in a subpopulation of both components. No i(12p) was identified in this case. The other combined tumor showed amplification in a minority of the seminoma cells only, and karyotyping failed. One of the nonseminomas, initially diagnosed as mixed nonseminoma showed no i(12p) by karyotyping. This tumor was xenografted orthotopically into a nude rat, and after about 10 months, a tumor completely composed of yolk sac tumor was formed. Karyotyping after direct harvesting revealed i(12p) in seven out of 14 metaphase spreads, confirmed by FISH (not shown). These metaphase spreads did not contain the 12p-amplification. However, a limited number of interphase nuclei isolated from the original tumor and the xenograft showed the presence of 12p-amplification by FISH (not shown). FISH on frozen tissue sections of these tumors demonstrated that less than 5% of the tumor cells from the primary tumor showed the 12p-amplification, preferentially in the embryonal carcinoma and yolk sac tumor component. This percentage was constant during subsequent xenograft-passages.

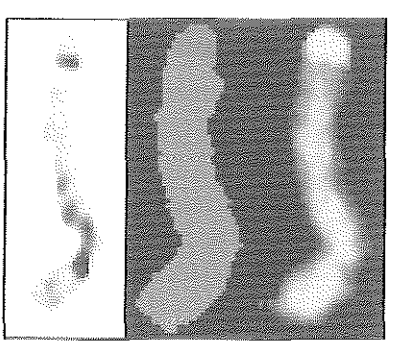
CGH was performed on three seminomas with homogeneous 12p-amplification, three of the heterogeneous nonseminomas and on both combined tumors. No other high level

amplifications, besides the one on 12p, were found in these cases, and a similar pattern of gains and losses was identified as reported before (Mostert *et al.*, 1996; Rosenberg *et al.*, 1999, not shown). Every tumor with 12p-amplification also showed gain of the complete short arm of chromosome 12, of which representative examples are illustrated in Figure 2A.

Fig.2 A: *Representative examples of comparative genomic hybridisation for chromosome 12 on a seminoma without a restricted 12p-amplification (left) and one with a homogeneous 12p-amplification (right). Note that the seminoma with the restricted 12p-amplification also shows gain of the complete short arm of this chromosome. The number of metaphase spreads analyzed is indicated.*

Spectral karyotyping was done on one seminoma, of which a sufficient number of metaphase spreads including the restricted 12p-amplification was available. The results confirm the presence of chromosome 12 material in the aberrant chromosome 12 derivatives, and in the most telomeric region a part of chromosome 20 (see Figure 2B).

Fig.2 B: *Representative example of banding (left panel) and spectral karyotyping (real color at the right and false color in the middle) of a chromosome 12 derivative known to contain restricted 12p-amplification (as determined by in situ hybridisation and CGH). Note that the chromosome is composed of chromosome 12 material (indicated in purple), except the most distal region of the p-arm, showing chromosome 20-specific material (indicated in blue).*



Restricted 12p-amplification and tumor progression

Matched CIS cells and micro-invasive seminoma of the TGCT with 12p-amplification were studied by FISH on tissue sections. The cells of interest were visualized by enzyme-histochemistry for alkaline phosphatase reactivity. Out of the eight seminomas studied, three showed the presence of both CIS and micro-invasive seminoma, and one only of CIS. The amplification was found in all micro-invasive components, but never in CIS, of which examples are shown in Figure 3. Also no restricted 12p-amplification was found in the matched CIS of one nonseminoma and one combined tumor. The micro-invasive seminoma cells adjacent to the combined tumor showed also a heterogeneous pattern of restricted 12p-amplification, as detected in the matched invasive tumor (not shown).

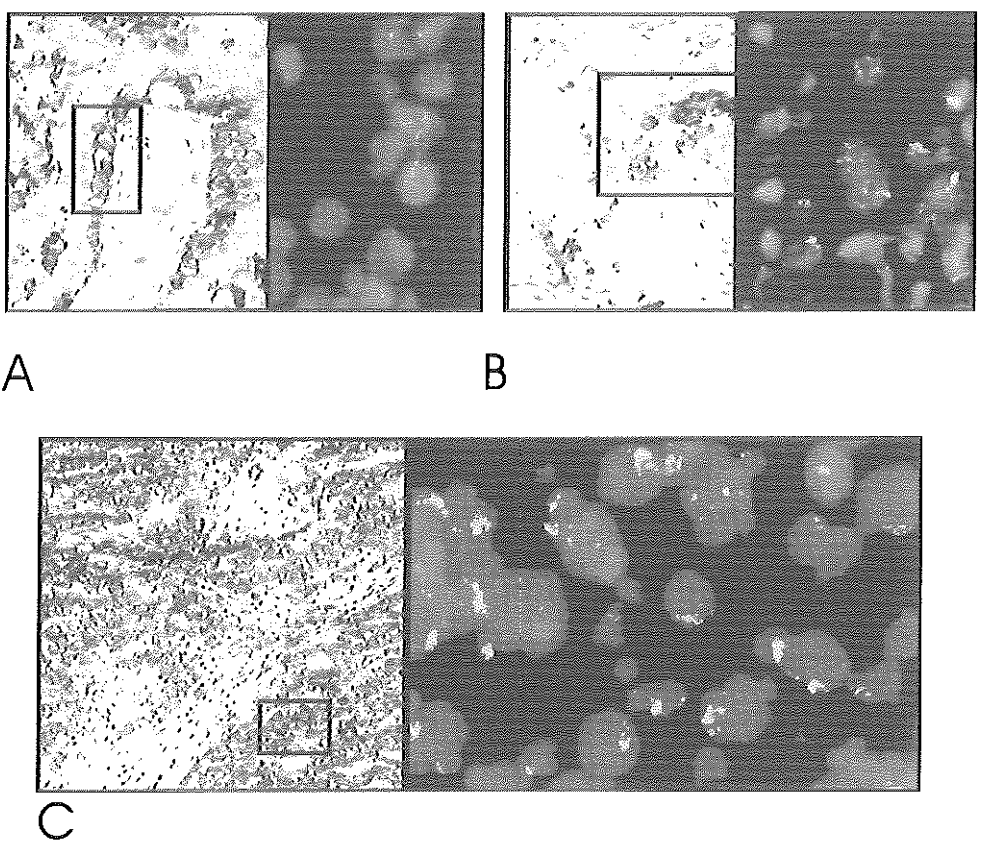


***Fig.3**: Representative examples of double fluorescent in situ hybridisation on frozen tissue sections of 4 μm using a probe specific for the centromeric region of chromosome 12 (red) and YAC#5 (green), mapped within the shortest region of overlap of amplification (see Figure 1). Shown are **A)** carcinoma in situ; **B)** micro-invasive seminoma; **C)** invasive seminoma. The tumor cells are identified by the direct enzymatic alkaline phosphatase detection method (stained in red) on a parallel tissue section.*

12p-amplification/RAS mutation, proliferation, apoptosis and in vitro survival

Immunohistochemistry for ki-67 on histological sections was performed to investigate the proliferation index in seminomas containing 12p-amplification (n=8)/*RAS* mutation (n=4, of two no approprioate material was available anymore), or neither of these (n=13). As indicated in Table 2, no differences between these groups were observed. However, all nine seminomas with 12p-amplification, as well as five out of the six with a mutated *RAS* gene, demonstrated low level of apoptosis in the primary tumor, indicated by the lack of profound DNA laddering after electrophoresis. In contrast, nine of the 13 tested seminomas without any of these features showed apoptosis (see Table 2 and Figure 4).

Table 2.

Summary of the results on proliferation index (prolif.index), apoptosis, and in vitro survival and volume of the tumor (cm^3) in seminomas with and without a restricted 12p-amplification/RAS mutation. (N = not available; +=present; -=absent) (standard deviations are indicated between brackets)

Wild-type *RAS*

		prolif. index	apoptosis		*in vitro* survival		tumor size
			+	-	+	-	
12p-ampl.	–	34.5 (10.5)	9	4	1	11	256 (173)
	+	29.7 (15.3)	0	9	4	0	246 (294)

Mutant *RAS*

		prolif. index	apoptosis		*in vitro* survival		tumor size
			+	-	+	-	
12p-ampl.	–	30.8 (3.1)	1	5	6	0	121 (88)
	+	N	N	N	N	N	N

The capacity of the tumor cells to survive *in vitro* after disruption of their micro-environment to generate single cell suspensions was studied, of which the results are also summarized in Table 2. We excluded that one of the newly identified seminomas in this study contained a mutation of either the N- or *K-RAS* gene in codons 12 and 13 (not shown). All six seminomas with a *RAS* mutation and all four tested seminomas with a restricted 12p-amplification showed prolonged *in vitro* survival (at least up to 10 days). Eleven seminomas without a restricted 12p-amplification or *RAS* mutation out of the 12 tested could not be maintained *in vitro*. Nine of these showed profound apoptosis. The seminoma that showed *in vitro* survival for several days did not show obvious apoptosis.

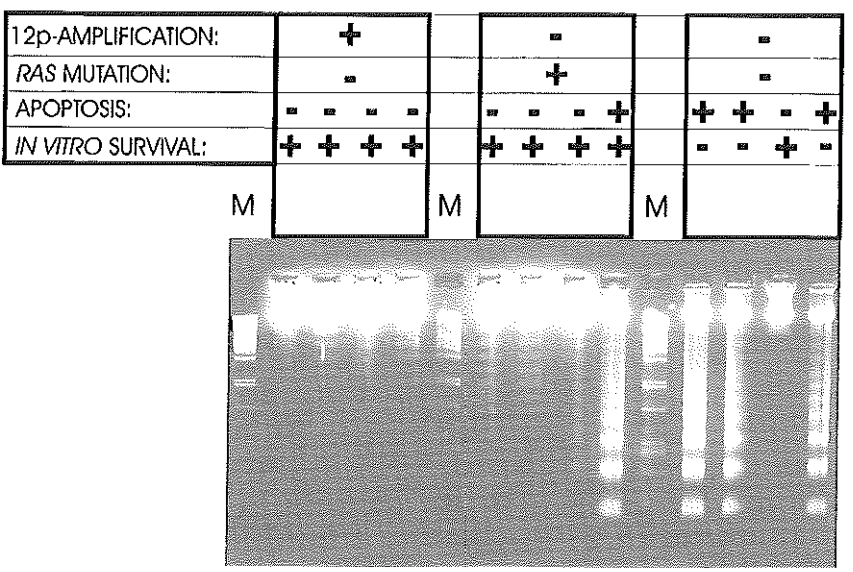


Fig.4: Representative examples of the presence of DNA laddering after electrophoresis (indicative for apoptosis) of 1 μg high molecular weight DNA isolated from primary seminomas with and without a restricted 12p-amplification/RAS mutation. Note the correlation between absence of apoptosis and presence of amplification or RAS mutation. In addition, the results of in vitro culture of the tumor cells are shown (see also Table 2).

The nonseminomas, with or without restricted 12p-amplification all lacked significant apoptosis, as was found for both combined tumors. All nonseminomas and one combined tumor were successfully karyotyped after short term *in vitro* culturing, indicating that these tumors allowed short term culturing *in vitro* (see **Materials and Methods**).

12p-amplification, RAS mutation, and clinical behaviour

The age at clinical diagnosis of patients with a seminoma with and without a restricted 12p-

amplification/*RAS* mutation is indicated in Figure 5. No age differences exist between seminoma and nonseminoma patients with and without a *RAS* mutation (Figure 5 and not shown). In spite of the small number of cases, seminoma patients with a restricted 12p-amplification showed a trend toward a younger age at clinical presentation than those without (p=0.055, Student's t test). When only the seminomas with a homogeneous pattern of 12p-amplification were included (n=6) (see above), the age difference was significant: 36.7 years without a restricted 12p-amplification, and 26.78 years with a restricted 12p-amplification (p= 0.023, Student's t test).

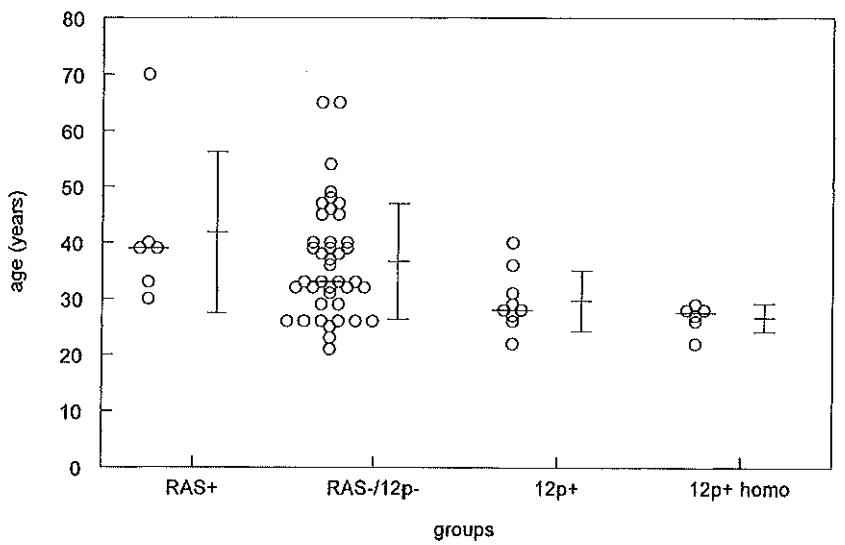


***Fig.5**: Schematical representation of the ages of patients at clinical presentation with a seminoma with and without a restricted 12p-amplification and RAS mutation (mean, average and standard deviations are indicated). In addition, the ages of the subgroup of patients with a homogeneous 12p-amplification are shown. No differences were found between the ages of patients with a seminoma without either of these aberrations and those with a RAS mutation. However, those with a 12p-amplification positive seminoma showed a borderline significant difference compared to the control group, while a significant difference was found in case only patients with a homogeneous 12p-amplification were included (p= 0.023). (Abbreviations used: RAS+ = RAS mutation; RAS-/12p- = no RAS mutation or restricted 12p-amplification; 12p+ = restricted 12p-amplification both heterogeneously and homogeneously present; 12p+ homo = homogeneous restricted 12p-amplification).*

No differences were found between these different groups regarding stage of the disease (they all presented as stage I or II), size of the tumor (see Table 2) and clinical response (all had a complete response). In addition, no correlation was found between outcome and age, stage, proliferation index, or apoptosis. Also no correlation was detected between the presence of 12p-amplification/*RAS* mutation and proliferation index.

To extend the analysis of the clinical significance of *K-RAS* mutations/12p-amplifications, we retrospectively investigated a series of 22 primary tumors and 33 surgical specimens from metastasis from a total of 44 different patients who failed cisplatinum-based chemotherapy. No *K-RAS* mutations were identified. The sensitive detection method (see **Materials and Methods**), makes it highly unlikely that mutated genes were obscured by a predominance of wild type alleles. A restricted 12p-amplification was detected once in a metastatic tumor (primary not available).

Discussion

Although gain of the short arm of chromosome 12 has already been recognized since more than 25 years as a characteristic chromosomal anomaly in TGCT (Atkin & Baker, 1983; Sandberg *et al.*, 1996, for review), the biological significance still remains unknown, and the clinical importance is a matter of debate. Because overrepresentation of 12p is found in all TGCT, mostly due to the presence of one or more isochromosomes of 12p (up to 80%) (Sandberg *et al.*, 1996; Van Echten-Arends *et al.*, 1995, for review), it is most probably a crucial step in the development of this cancer. Amplification of a restricted region of 12p on top of gain of the complete short arm of chromosome 12 was for the first time found in a metastatic seminoma (Suijkerbuijk *et al.*, 1994). Subsequently, we and others showed that this anomaly can also be present in primary TGCT (Mostert *et al.*, 1996; Korn *et al.*, 1996; Mostert *et al.*, 1998, and this study). Here we show that the actual incidence of a restricted 12p-amplification is around 8% for primary TGCT. Our earlier observation that the three known genes *JAW1, SOX5,* and *K-RAS* map in this region (Mostert *et al.*, 1998), is confirmed in the study presented here. We hypothesized, that if important genes for the development of this cancer reside in this region of 12p, a comparative study of TGCT with and without this type of amplification, could be meaningful. In addition, because *K-RAS* maps within the SROA, these tumors were compared with previously published seminomas with a *RAS* mutation (Olie *et al.*, 1995a).

Although we investigated nine additional TGCT with a restricted 12p amplification compared to our previous study (Mostert *et al.*, 1998), we were unable to further reduce the SROA. However, it was found that the breakpoints of the amplicon cluster in rather narrow regions: 40% between D12S1313 and *K-RAS* at the distal end, and 53% between AFM267yc9 and D12S1945 at the proximal end. The total length of the short arm of chromosome 12 is expected to be around 40Mb. Assuming that the STSs are spread over the genome with intervals of about 250 kb, 160 fragments are to be expected. The chance that one breakpoint maps within the region between AFM267yc9 and D12S1945 is estimated to be 6.3E-3. The change that six additional breakpoints map to that particular region is around 5.7E-11. In addition, the chance that six breakpoints map between *K-RAS* and D12S1313 is around 1.3E-11 (binomial distribution). This indicates a strong positive selection towards these particular regions. This could be due to the presence of fragile sites, although they have not been identified at these regions. The closest fragile site on chromosome 12 has been reported on 12p11 (Ford, 1981), which might be related to the development of the isochromosome 12p. The borders may alternatively be determined by the presence of genes which confer selective advantage to the tumors. If this is true, the amplicon contains at least two genes of interest, a hypothesis which is currently under investigation.

All TGCT with 12p-amplification identified so far also show gain of the complete short arm of chromosome 12. Interestingly, seminomas with a homogeneous 12p-amplification, i.e., present in all cells, lack i(12p), while those showing a heterogeneous pattern could contain an isochromosome. The restricted 12p-amplification and i(12p) were never found in the same cell. A similar pattern was found for the nonseminomas and combined tumors. Interestingly, the

seminomatous components of the combined tumors showed a similar pattern of restricted 12p-amplification as the pure seminomas. Clearly, 12p-amplification is found predominantly in TGCT without i(12p). These results suggest that there are at least two mechanisms leading to extra copies of the short arm of chromosome 12 in TGCT: i(12p) formation, and an alternative way, also leading to extra copies of the complete short arm of chromosome 12, which can be followed by amplification of a restricted region of 12p.

The homogeneous presence of 12p-amplification in most of the identified seminomas (and not in nonseminomas) implies that increased copy numbers of genes present in the amplified region result in a selective advantage for these tumor cells containing this specific anomaly. We demonstrated that this is related to a reduced apoptosis, and not due to enhanced proliferation. A similar lack of apoptosis was observed previously by us in seminomas containing a mutated *RAS* gene (Olie *et al.*, 1995b; Oosterhuis *et al.*, 1997), which also showed an enhanced *in vitro* survival and reduced apoptosis. These results are in accordance with the idea that activation of *RAS* by mutation or amplification can cause inhibition of apoptosis.

In spite of data on the role of activated *RAS* in resistance to irradiation and chemotherapy, and induction of metastatic potential (Ananthaswamy *et al.*, 1989; Holford *et al.*, 1998; Isonishi *et al.*, 1991; Kinashi *et al.*, 1998; Sklar, 1988a; Sklar, 1988b; Takiguchi *et al.*, 1992), and our results on *in vitro* survival and apoptosis (Olie *et al.*, 1996; Olie *et al.*, 1995b), no differences were found regarding stage of the primary seminoma at clinical presentation and treatment response based on the presence of a *RAS* mutation or restricted 12p-amplification. This is supported by an observation in two patients (Rao *et al.*, 1998): the restricted 12p-amplification (detected by CGH) was present in both a responding and a nonresponding tumor. In addition, we observed no predictive value of *K-RAS* mutations and restricted 12p-amplification in chemo-resistance of patients with extensively pretreated TGCT. Patients having a seminoma with a homogeneous restricted 12p-amplification were, however, younger at clinical presentation than those lacking it. This is most likely explained by an early development of the restricted 12p-amplification in these tumors, leading to reduced apoptosis. This combined with an unchanged proliferation index results in faster growth of the tumor and an earlier clinical presentation. Such an age difference was not observed in the case of a *RAS* mutation, which is likely because mutation of *RAS* is a relatively late event in the pathogenesis of TGCT. This is supported by our observation that TGCT with a mutated *RAS* gene also show gain of the complete short arm of chromosome 12, although no information about the presence of i(12p) is available (not shown).

Our data suggest that *RAS* mutation and restricted 12p-amplification are related to progression of TGCT. It allows tumor cells to survive outside the specific micro-environment of the CIS cells by inhibition of induction of apoptosis, known as "anoikis" (Frisch & Francis, 1994). The reason that homogeneous 12p-amplification is predominantly found in seminomas can be related to the diminished requirements of nonseminomatous tumor cells for survival. This idea is supported by the relative ease of short term culture for karyotyping (Van Echten-Arends *et al.*, 1995), and the success rate of establishing nonseminoma cell lines (Andrews *et al.*, 1996). It

remains to be established whether involvement of *RAS* causes down-regulation of FAS, as suggested recently (Fenton *et al.*, 1998; Peli *et al.*, 1999), and whether activation of the serine/threonine kinase AKT is involved (Aoki *et al.*, 1998; Brunet *et al.*, 1999). In addition, it is unknown thus far, which genes in the SROA are in fact involved. The absence or at least lower incidence of gain of 12p sequences in CIS as reported before (Looijenga *et al.*, 1999; Vorechovsky & Mazanec, 1989; Vos *et al.*, 1990 and unpublished observations) support this model.

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Chapter 8

General Discussion

General Discussion

Until recently, metaphase spreads were required for the analysis of the chromosomal constitution of neoplasms. This requirement was partly circumvented by the introduction of FISH (Pinkel et al. 1986). Ever since the method has been improved and modified. CGH introduced by Kallioniemi in 1992, is an entirely novel application of FISH (Kallioniemi et al. 1992; Du Manoir et al. 1993). This technique visualises chromosomal gains and losses compared to the average ploidy by hybridising normal and tumor DNA with normal metaphase spreads. FISH and CGH have created new opportunities for identification of chromosomal anomalies in tumors, because they can be applied on frozen and paraffin-embedded archival tissue.

Mainly due to low incidence, little is known about the chromosomal constitution of the varous types of germ cell tumors. A more thorough knowledge of the chromosomes in the different types of germ cell tumors could shed light on their relationship and the genes involved in their pathogenesis. With this aim, we did karyotyping, FISH and CGH on a series of human testicular germ cell tumors of neonates and infants (GCTI), adolescents and young adults (TGCT) and elderly men (SS). In the following paragraphs the contribution of the findings to the understanding of the pathogenesis of these tumors will be discussed, including the role of gain of 12p.

Neonates and infants

In this particular group of germ cell tumors one of the striking findings is that histologically they are either teratomas or yolk sac tumors, and rarely a mixture of both (Mostofi and Sesterhenn 1985; Harms and Janig 1986). CIS has not been found in these tumors with some questionable exceptions (Jørgensen et al. 1995; Jenderny et al. 1995; Chapter 2). So far, most of the teratomas have been investigated with flow cytometry and/or karyotyping. Using these methods, no aberrations have been observed (Kaplan et al. 1979; Silver et al. 1994; Stock et al. 1995), which theoretically may be related to loss of tumor cells during processing of the samples. To exclude this problem, we did CGH on microdissected teratomatous elements, and confirmed the absence of gross chromosomal anomalies. In addition, FISH showed that these tumors are diploid.

In contrast, aneuploidy has been reported for the yolk sac tumor (Kommoss et al. 1990; Silver et al. 1994). Karyotyping and FISH demonstrated loss of 1p36, 6q and aberrations of 3p (Oosterhuis et al. 1988; Oosterhuis et al. 1993; Perlman et al. 1994; Jenderny et al. 1995; Stock et al. 1995; Perlman et al. 1996; Bussey et al. 1999). Although we could not confirm loss of 1p36 by CGH, FISH showed that this particular region is indeed underrepresented in all yolk sac tumors studied. In total, we performed CGH on four yolk sac tumors of the infantile testis. In spite of this limited number of cases, a rather consistent pattern of chromosomal imbalances was found. The following imbalances (involved bands are between brackets) were detected: loss

of parts of 4q (23-33) and 6q (16-22), and gain of parts of 20q was found in all cases, and loss of parts of 1p (21-31), 8q (23) and gain of parts of 3p (22-24), 9q (34), 12p (12-13), 17q (22-25), 19q (13) and 22 (13) in at least three cases (see also Table 1):

Table 1;

Chromosomal aberrations in testicular germ cell tumors found by CGH in at least 50% of the cases of yolk sac tumors (YS) of infants and spermatocytic seminomas (SS) (Mostert et al, submitted; Rosenberg et al. 1998) respectively, and in at least two of the five studies on seminomas (SE) and nonseminomas (NS) (Korn et al. 1996; Mostert et al. 1996; Ottesen et al. 1997; Summersgill et al. 1998; Rosenberg et al. 1999) as depicted in Figure 1. Aberrations present in all cases of a specific group are presented in bold and those unique for a particular entity are underlined. (Only the p or q arm is indicated, not the specific regions involved).

Group	histology	Loss	Gain
Neonates & infants	**YS**	1p, **4q**, **6q**, 8q, Xp	3p, 9q, 12p*, 17, 19, **20q**, Y
adolescents & young adults			
	SE	1p, 4, 5, 9p, 11, 13, 16p, 17p, 18	1q, 2, 3p, 6p, 7, 8, 10, **12p**, 12q, 14, 15, 20, 21, 22, X
	NS	4, 5, 6q, 10, 11q, 13, 18	1, 2, 6q, 7, 8, **12p**, 12q, 14 15, 16, 17q, 20q, 21, X
elderly	**SS**	13, 15, 22	1q, **9**, 18, 20

*only 12p12-p13 is involved

Besides the four yolk sac tumors, we also studied a recurrent yolk sac tumor of a sacral teratoma. A highly similar pattern of chromosomal aberrations, both gains and losses, was found compared to those of the infantile testis. This is in agreement with earlier karyotypic

findings (Perlman et al. 1994; Bussey et al. 1999), and indicates that histology rather than anatomical site determines the genetic constitution of these tumors.

One of the yolk sac tumors of the infantile testis showed a high level amplification of the 12q12-q14 region, known to contain the *MDM2* gene. MDM2 is described to form a complex with P53, resulting in degradation thereby inhibiting the function of P53 (Haupt et al. 1997; Lane and Hall 1997; Midgley and Lane 1997). This led us to investigate the presence of P53 and MDM2 in these tumors using immunohistochemistry. While all teratomas and yolk sac tumors showed the presence of MDM2, no P53 was observed. Accordingly, no mutations within exons 5-8 of *P53* were found. These data indicate that *P53* is not genetically inactivated in these tumors but might be non-functional (Prives 1998).

Because of the absence of chromosomal abnormalities in teratoma, our data do not give new insights about the possible relationship between teratoma and yolk sac tumors. It remains to be proven whether teratoma can progress into yolk sac tumor, as has been suggested for teratoma of the sacral region (Gonzalez-Crussi 1982) and supported by findings in a mouse model (Van Berlo et al. 1990).

Adolescents and adults

TGCT comprising SE and NS, have been studied extensively with flow cytometry, karyotyping and/or FISH. So far the data show that SE and NS are consistently aneuploid, with a specific pattern of relative gains and losses, (Van Echten-Arends et al. 1995; Sandberg et al. 1996, for review). In fact, SE, like CIS, are hypertriploid, and NS hypotriploid. Because of the need of *in vitro* culturing of cells of NS for karyotyping, selection might occur. In addition, this can also be the case for SE which are analysed after direct harvesting of metaphase spreads from the tumor. Therefore, TGCT have also been investigated by CGH recently (Korn et al. 1996; Mostert et al. 1996; Ottesen et al. 1997; Summersgill et al. 1998; Rosenberg et al. 1999). In Figure 1, the results of the different published CGH studies are summarised and in Table 1 the most occurring chromosomal aberrations found are indicated. Overall, the findings support the information obtained with karyotyping, i.e. gains of chromosomes 7, 8, 12 and X and losses of chromosomes 4, 5, 11, 13, 18 and Y (Van Echten-Arends et al. 1995). Furthermore, all CGH studies confirm the presence of extra copies of the complete short arm of chromosome 12. Our initial study (Mostert et al. 1996) showed the lowest number of anomalies, which can be explained by the limitations of the software used.

Because SE have an intermediate DNA content between 3 and 4n and NS between 2 and 3n, the used average ploidy in CGH to detect imbalances does not correspond to any of the ploidy fraction of the tumor genome. This problem has been solved recently using multiple normalisations besides the average as normally applied (Rosenberg et al. 1997; Rosenberg et al. 1999). The modified analysis showed additional numerical aberrations which are usually not detected by conventional CGH: gain of chromosome 1, 2, 6, 14, 15 and 21 and loss of chromosome 9. Again, this approach showed that gain of 12p is consistent, even higher than the

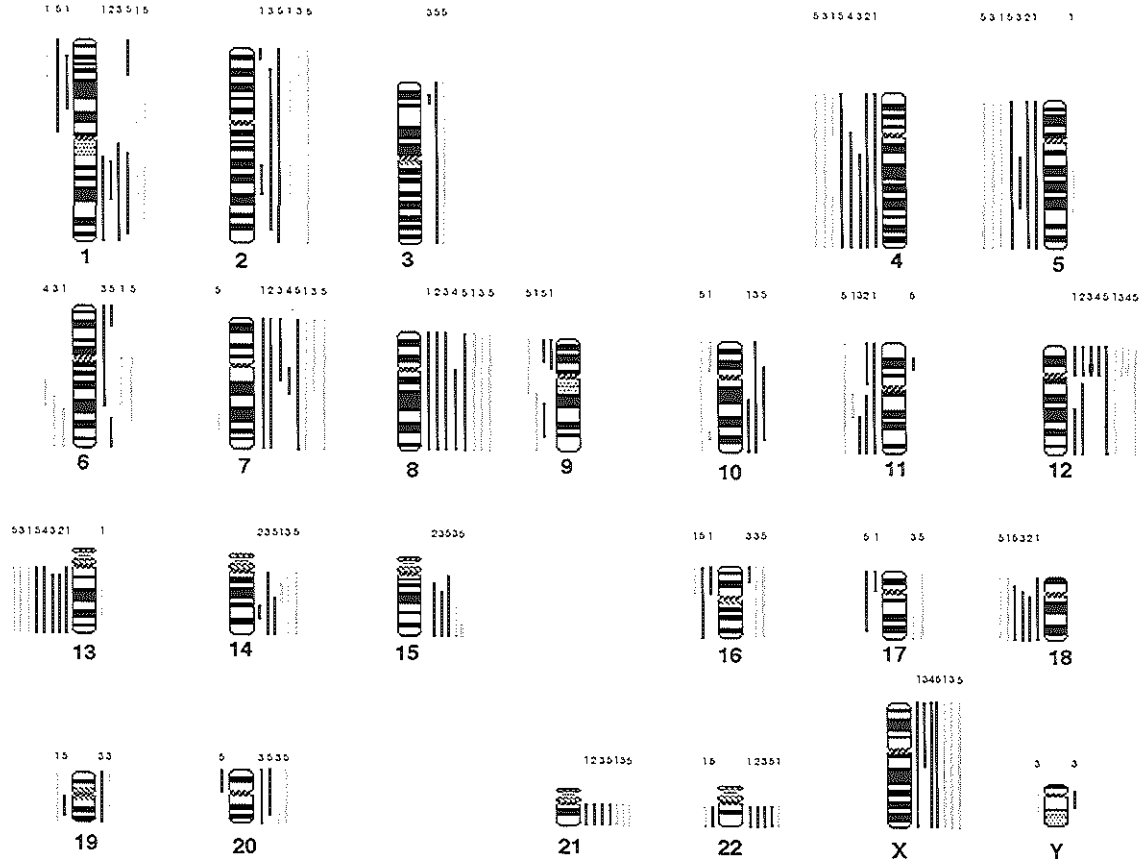

Fig. 1:Overview of the chromosomal gains and losses of primary testicular germ cell tumors of adolescents and adults as determined by five independent comparative genomic hybridization-studies (1: Summersgill et al.,1998; 2: Ottesen et al., 1997; 3: Korn et al., 1996; 4: Mostert et al. 1996; 5: Rosenberg et al., 1999). Gains and losses are indicated at the right and left side of the chromosomes, respectively. Seminomas are shown in black and nonseminomas in gray. The anomalies shown are those which are present in at least 25% of the tumors in that particular study. In total 43 seminomas and 31 nonseminomas have been investigated.

upper-ploidy level in the majority of cases.

Besides a highly similar pattern of chromosomal gains and losses in SE and NS, several differences have been observed both using CGH and karyotyping (Van Echten-Arends et al. 1995; Summersgill et al. 1998; Rosenberg et al. 1999). For example, loss of chromosome 10 was only found in NS, while loss of (parts of) chromosome 16 and 17 was mainly seen in SE. In addition, chromosomes 15 and 22 are present in higher numbers in SE than in NS (De Jong et al. 1990, see Figure 1). These differences might explain the hypertriploid versus hypotriploid DNA content for SE and NS, respectively (Oosterhuis et al. 1989).

Elderly

The first information about the chromosomal constitution of SS became available in 1973, showing a modal chromosome number of two cases, containing 52 and 82 chromosomes, respectively (Atkin 1973). Subsequently, several investigators studied the ploidy of these tumors either by flow cytometry or image analysis, resulting in conflicting data (Talerman et al. 1984; Dekker et al. 1992; Takahashi 1993; Looijenga et al. 1994). We reported the first karyotype of a SS, showing an aneuploid chromosome number and a limited amount of structural aberrations (Rosenberg et al. 1998). In addition, this sample and three others, including bilateral tumors, were studied with CGH, indicating that gain of chromosome 9 was the only consistent anomaly (Rosenberg et al. 1998, see Table 1). The finding that most imbalances involved whole chromosomes, might be associated with their relatively benign behaviour. *In situ* hybridisation with probes specific for the X and Y chromosomes revealed a heterogeneous population of tumor cells. This might be related to the presence of small, intermediate and large cells in the histology (Burke and Mostofi 1993; Cummings et al. 1994; Eble 1994; Looijenga et al. 1994). The relationship between these particular cell types remains to be established. Microdissection in combination with CGH is going to be a useful tool to answer this question (Looijenga et al. 1999).

SS are supposed to originate from type B spermatogonia (Masson 1946; Rosai et al. 1969; Romanenko and Persidskii 1983). The finding of common chromosomal abnormalities in the bilateral SS might challenge this idea. Possible explanations for these similarities are a germ line mutation (*de novo* or familial) or the presence of a metastases. Both ideas seem unlikely because no familial SS have been reported and metastases have never been found in pure SS. Recently, however, another hypothesis was put forward. Based on the finding of c-KIT in SS it has been suggested that at least a percentage of SS originate from early (primordial) germ cells (Kraggerud et al.1999). This might implicate that mutation(s) take place before migration to the gonadal anlages. A relationship between SE and SS has also been suggested based on the positivity of these tumors for the nuclear antigen Ki-A10 (Rudolph et al. 1999). Analysis of SE and SS for expression of stage specific and meiosis related genes might be informative.

Pathogenetic relationship(s)

It is generally agreed upon that SS does not originate from CIS, although an early (primordial) origin seems to be possible (see above). The discussion regarding separate or common pathogenetic relationships of GCTI and TGCT is partly due to the finding of CIS-like cells in the adjacent parenchyma of the first. In fact, two case reports showed these cells based on PLAP-positivity and morphology (Jacobsen 1992; Stamp et al. 1993), although these findings were criticized by others (Parkinson and Ramani 1993; Hawkins and Hicks 1998). Of notice is that immunohistochemistry cannot be used to identify CIS cells in testicular parenchyma in the first year of life since normal parenchyma may contain PLAP positive germ cells (Jørgensen et al. 1993). In addition, a common pathogenetic pathway of GCTI and TGCT seems unlikely, because of their difference in ploidy (see above and Figure 2).

Interesting however is the finding that yolk sac tumor of the infantile testis may contain extra copies of the complete 12p region (Stock et al. 1995; Jenderny et al. 1996), sometimes even an i(12p) detected by FISH (Stock et al. 1995), although karyotyping could not confirm this (Perlman et al. 1994). This finding of extra copies of the complete 12p region was not found in our limited series of cases. We found gain of a restricted part of 12p in three of the five yolk sac tumors (Figure 2 and Chapter 2), but this region is different from the region amplified in TGCT (Chapter 5). Most likely one is dealing with a TGCT from a patient with early puberty in case an i(12p) is present.

Additional support for an independent pathogenesis of GCTI and TGCT comes from both epidemiological and immunohistochemical data. In contrast to TGCT, no rising incidence is observed for GCTI. While P53 is reported to be present in TGCT (Schenkman et al. 1995; Guillou et al. 1996), this protein was not found in GCTI (Chapter 2).

Moreover, putative animal models for GCTI and SS have been identified. Mouse teratocarcinomas most probably are models for GCTI (Walt et al. 1993), showing a similar capacity of progression to yolk sac tumor as their human counterparts (Van Berlo et al. 1990). Canine seminomas most likely are the counterpart of human SS (Looijenga et al. 1994). The absence of animal models for TGCT underscores their uniqueness of these tumors for men.

In conclusion, these data and the CGH studies reported here are strongly in favour of separate genetic pathways for the GCTI, TGCT and SS (see Figure 2).

12p-amplification in TGCT

All TGCT show gain of the complete short arm of chromosome 12, illustrated by karyotyping, FISH and CGH (see above). In the majority of cases this is due to the presence of i(12p). FISH using a probe specific for the centromere, has been reported as a tool to detect i(12p) in interphase nuclei (Mukherjee et al. 1991; Rodriquez et al. 1992). We demonstrated that this method is not reliable, although it can be improved using double FISH, i.e., combination of two probes, specific for the centromere and for the short arm of chromosome 12 (Chapter 4). In addition, a combination of paint probes specific for the short and long arm of

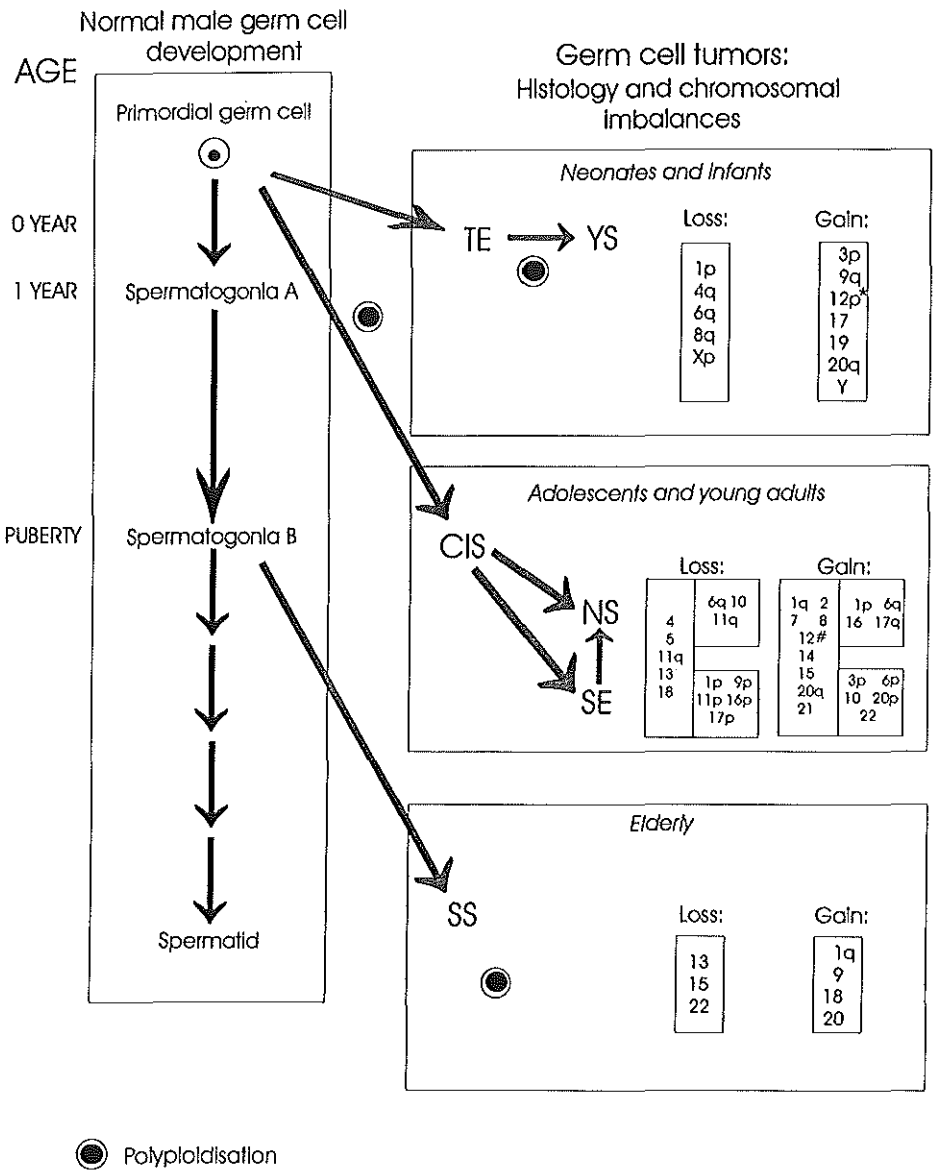

Fig.2: Schematic representation of the development of normal male germ cells (left panel) and testicular germ cell tumors (right panel). The existence of three separate pathogenetic pathways for germ cell tumors of neonates and infants, adolescents and young adults, and elderly are supported by their chromosomal constitution. (Abbreviations used; TE= teratoma; YS= yolk sac tumor; CIS= carcinoma in situ; NS= nonseminoma; SE= seminoma; SS= spermatocytic seminoma).

** affecting the 12p12-p13 region; # predominantly affecting the complete 12p region.*

chromosome 12 was also successfully applied for this purpose (Blough et al. 1997; Blough et al. 1998).

Although the formation of an isochromosome 12 is not the initial step in the pathogenesis of TGCT (Geurts van Kessel et al. 1989), its consistent presence in invasive tumors indicates that extra copies of genes on the short arm of chromosome 12 are crucial in the development of this cancer. Identification of the involved genes is however difficult, because the short arm of chromosome 12 is 40 Mb containing as many as 850-1400 genes (http://www.ncbi.nlm.nih.gov/genemap). Interestingly, we and others identified a number of TGCT with an amplification of a restricted region of 12p (Suijkerbuijk et al. 1994; Korn et al. 1996; Mostert et al. 1996). We hypothesized that if important genes reside within the amplified region, a comparative investigation of TGCT with and without such an amplification can give a clue regarding the biological role of gain of 12p in the development of TGCT. Therefore, we studied the incidence of the restricted 12p amplification in a non-selected series of primary consecutive TGCT. The 12p amplification was found in around 8% of the cases, both affecting SE and NS (Chapter 7). This amplification was predominantly found in cases without i(12p), suggesting it to be an alternative mechanism to gain 12p-sequences.

FISH showed that the presence of the restricted 12p amplification is related to invasive growth. It was found in the invasive tumor and micro-invasive SE cells, but not in CIS. Interestingly, it was homogeneously present in most SE, suggesting that it results in a selective advantage for either growth or inhibition of apoptosis in these particular cells. This latter possibility was supported by analysis of DNA laddering (Olie et al. 1996) (Chapter 7) and *in vitro* survival (Chapter 7). Furthermore, SE with a homogeneous restricted 12p-amplification presented at a significantly earlier age than those without, although it was not correlated with the stage of the disease at clinical presentation or treatment response. Similarly, restricted 12p-amplification has no prognostic or predictive value in NS.

In spite of identification of a relative large series of TGCT with a 12p amplification (Chapter 7), the shortest region of overlap of amplification (SROA) was not found to be smaller than initially determined (Chapter 6). Noteworthy, the breakpoints of the amplicons seem to cluster in particular regions which might suggest involvement of multiple genes in this particular region. This model is currently under investigation. Thus far three genes have been mapped within the SROA: *SOX5, JAW1* and *K-RAS*. Although we do not exclude other genes to be involved, we currently favour *K-RAS* as at least one of the candidate genes because of similar *in vitro* behaviour of SE with a *RAS* mutation (Olie et al. 1995). Mutations of *K-RAS,* like 12p amplification, has no clinical value.

Future perspectives

The studies described in this thesis have contributed to the understanding of the (molecular) cytogenetics of human testicular germ cell tumors. Obviously, many questions remain to be answered, in particular related to the factors (genes) involved in the early development of these cancers. Investigation of families with a predisposition for a specific type of cancer appears to be very fruitful in the elucidation of its molecular mechanisms. For TGCT is has been proposed that about 30% of all the patients have a genetic predisposition (Nicholson and Harland 1995) and the search for predisposing genes in these tumors resulted in suggestive evidence for parts of chromosomes 1, 3, 4, 5, 12, 14, 18 and X (Leahy et al. 1995; Rapley et al. 1998; Rapley et al. 2000). To obtain more evidence for the presence of predisposing genes, more families need to be studied or alternative approaches must be exploited. If these turn out to be tumor suppressor genes, these results have to be compared with loss of heterozygosity studies.

The biological role of gain of genes on 12p in the development of TGCT remains to be resolved. In this context analysis of (low) copy numbers of 12p in CIS, and the possible increase during the early phase of invasive growth is of great interest. Final proof about the involvement of candidate genes awaits the development of appropriate assays *in vitro* or in animal models.

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Summary/samenvatting

Summary

Germ cell tumors are a heterogeneous group of tumors with an incidence between 0.1-8/100.000. They occur mainly in the gonads, but also in extragonadal sites. In the male, the most occurring site of origin is the testis. Three groups of testicular germ cell tumors can be distinguished:

- germ cell tumors of neonates and infants (GCTI): teratomas and yolk sac tumors
- testicular germ cell tumors of adolescents and young adults (TGCT): seminomas and nonseminomas
- testicular germ cell tumors of the elderly man (SS): spermatocytic seminomas

This thesis describes the study of chromosomal aberrations in these three groups of testicular germ cell tumors by karyotyping, fluorescence *in situ* hybridisation (FISH) and comparative genomic hybridisation (CGH). Because of their rarity (0.1-2/100.000), the genetic information of especially the GCTI and SS is limited. **Chapter 2** describes the chromosomal analyses of GCTI. The results confirm previous indications for the gross absence of chromosomal aberrations in teratomas (TE). In contrast, the yolk sac tumors (YST) show a consistent pattern of chromosomal abnormalities; loss of (parts of) 1p, 4q, 6q and gain of parts of 3p, 9q, 12p, 17, 19q, 20q and 22. Immunohistochemistry shows presence of MDM2 and absence of P53 in all studied GCTI. In **chapter 3** the chromosomal constitution of four SS, including one bilateral, is investigated. These tumors show mainly numerical aberrations, with gain of chromsome 9 as only consistent aberration. FISH illustrate that SS are heterogeneous, supporting histological findings.

The TGCT always show overrepresentation of the short arm of chromosome 12; in 50-80% as an isochromosome. **Chapter 4** describes the detection of this aberration in cell-lines of TGCT using a bi-colour FISH approach. A series of primary TGCT was analysed with CGH and described in **chapter 5**. Besides the conformation of earlier karyotypic data, this study identifies the presence of a small amplicon on the short arm of chromosome 12, previously described only in a metastasis of TGCT. **Chapter 6** describes the detection and mapping of the shortest region of overlap of amplification (SROA) on 12p. In the SROA, estimated between 1.7-3.2 Mb in size, three known genes are mapped; *K-RAS, SOX5* en *JAW1*. In an attempt to diminish the SROA, a series of TGCT (n=76) was investigated for the presence of 12p-amplicons (**chapter 7**). Despite the fact that 12p amplicons are found in about 8% of these tumors, affecting both seminoma and nonseminoma, the SROA is not reduced. The breakpoints of the amplicons are found to cluster in particular regions which might suggest the involvement of multiple genes in the amplified region.

FISH indicates that 12p-amplification arise rather early during tumor development, associated with invasive growth. Seminomas with a restricted 12p amplification show, like tumors with a *RAS* mutation, a better *in vitro* survival. This finding together with the absence of DNA-laddering, suggest a blockade of apoptosis in these tumors. The *in vitro* similarities of tumors with 12p amplicon and those with a mutation in *RAS* are in favour for *K-RAS* as one of the candidate genes on 12p. Despite the fact that seminomas with 12p amplification in all tumorcells are manifested clinically at a significantly younger age, no other clinical value was found. **Chapter 8** describes the pathogenetic relationship between the different groups of testicular germ cell tumors. The differences in ploidy, chromosome pattern, presence or absence of CIS and immunohistochemistry, support a separate pathogenesis for each group of testicular germ cell tumors.

Samenvatting

Dit proefschrift beschrijft het onderzoek naar afwijkingen in het DNA van kiemceltumoren van de testis. Om dit onderzoek te kunnen begrijpen volgt eerst enige basale informatie over DNA en kanker.

De erfelijke informatie van iedere cel bevindt zich in het DNA, gelegen in de celkern. Bij delende cellen is het DNA sterk gecomprimeerd en zichtbaar in de vorm van speciale structuren: de chromosomen. Alle menselijke cellen, met uitzondering van de geslachtscellen, bevatten 23 paar chromosomen. Soms komen afwijkingen in deze chromosomen voor, zoals bij kankercellen. Deze afwijkingen kunnen spontaan ontstaan of door verschillende externe factoren zoals bijvoorbeeld virussen of bepaalde stoffen in voeding en omgeving, en kunnen onder speciale condities worden overgeërfd. Afwijkingen in chromosomen of DNA kunnen aanleiding geven tot het ontstaan van kanker, een proces dat zich meestal uitstrekt over een periode van tientallen jaren.

Het aantonen van deze afwijkingen kan van nut zijn bij het identificeren en het voorspellen van het gedrag van een kanker. Bovendien kunnen bepaalde afwijkingen een indicatie geven over welke genen betrokken zijn bij de ontwikkeling ervan. Kanker wordt veelal gekenmerkt door ontregeling van één of meer genen. Dit kunnen genen zijn die normaal gesproken de groei remmen (suppressorgenen), genen die groei stimuleren (oncogenen) of genen die schade in het DNA herstellen (DNA repair-genen).*

Genetische afwijkingen kunnen op chromosomaal niveau zichtbaar zijn. Dit kan aangetoond worden door de chromosomen, verkregen uit delende cellen, te behandelen met speciale kleuringstechnieken. Meer recent ontwikkelde technieken als FISH (fluorescence in situ hybridisation) en CGH (comparative genomic hybridisation) kunnen zelfs bij afwezigheid van delende cellen, chromosomale afwijkingen in kaart brengen. Dit is vooral van belang bij solide tumoren waarvan delende cellen vaak moeilijk te verkrijgen zijn. In dit proefschrift werden deze technieken toegepast voor de analyse van chromosomale afwijkingen van de (solide) kiemceltumoren van de testis.

Kiemceltumoren zijn relatief zeldzaam met een incidentie van 0.1-8 per 100.000. Zij ontstaan uit kiemcellen, welke de voorlopers zijn van de zaadcellen bij de man en de eicellen bij de vrouw. De tumoren die uit deze cellen ontstaan kunnen op verschillende plaatsen voorkomen. Bij de man is de testis de meest voorkomende locatie. Binnen de groep van testiculaire kiemceltumoren worden drie groepen onderscheiden:

- die van het jonge kind (GCTI): teratomen (TE) en dooierzak tumoren (YS)
- die van adolescenten en jonge volwassenen (TGCT): seminomen en nonseminomen
- die van de oudere man (SS): spermatocytair seminomen

Door de chromosomale samenstelling van deze drie groepen te onderzoeken kan kennis worden verkregen over welke (genetische) afwijkingen betrokken zijn bij hun ontwikkeling. Het vergelijken van deze groepen onderling, geeft informatie over de mogelijke pathogenetische relatie(s) tussen de verschillende tumortypen.

De GCTI en SS zijn het meest zeldzaam (0.1-0.2 per 100.000.). Over de chromosomale samenstelling van deze tumoren is dan ook nog maar weinig bekend. Met behulp van de technieken FISH en CGH werd voor beide groepen een karakteristiek chromosoompatroon

gevonden. De GCTI vertonen een aantal consistente chromosoom afwijkingen: verlies van delen van chromosoom 1, 4 en 6 en winst van delen van chromosoom 3, 9, 12, 17, 19, 20 en 22. Bovendien kon aannemelijk worden gemaakt dat het (proto) oncogen *MDM2* een rol zou kunnen spelen in de ontwikkeling van deze tumoren. Bij de SS werden voornamelijk afwijkingen van complete chromosomen gevonden met winst van chromosoom 9 als enige consistente afwijking.

Doordat de incidentie van de TGCT een stuk hoger is dan die van de andere twee groepen nl. 6-8 per 100.000, zijn deze tumoren veelvuldiger bestudeerd, inclusief hun chromosomale patroon. TGCT bezitten altijd extra kopieën van het bovenste deel (de korte arm) van chromosoom 12, meestal in de vorm van een zogenaamd isochromosoom. Dit wijst erop dat genen op de korte arm van chromosoom 12 van belang zijn voor de ontwikkeling van TGCT. Identificatie van deze genen is echter moeilijk omdat theoretisch 2000-4500 genen in dit gebied kunnen liggen. Met behulp van de CGH techniek was het mogelijk in ongeveer 8 % van de primaire TGCT een amplificatie** van een klein gebied van de korte arm van chromosoom 12 (12p) te detecteren. Hierdoor waren we in staat het gebied van studie op 12p te verkleinen en een aantal genen te selecteren die van belang zijn voor de biologie van TGCT. Tot nu toe lijkt het oncogen *K-RAS* de meest voor de hand liggende kandidaat. Deze conclusie wordt ondersteund door het feit dat in weefselkweek, tumoren met een 12p-amplificatie hetzelfde gedrag vertonen als tumoren met een mutatie van *K-RAS*. Om meer informatie over de biologische rol van de 12p-amplificatie te verkrijgen, werden tumoren met en zonder amplificatie met elkaar vergeleken. Seminomen met een 12p-amplificatie manifesteren zich op significant jongere leeftijd (ca. 27 jaar) dan seminomen zonder 12p-amplificatie (ca. 35 jaar). Desondanks kon geen klinische betekenis van de 12p-amplificatie worden vastgesteld.

De gevonden verschillen in chromosoompatroon bij de drie groepen testiculaire kiemcel tumoren ondersteunen de gedachte dat de tumoren van deze drie groepen verschillende, onafhankelijke ontstaanswijzen hebben en dat hierbij verschillende genen een rol spelen.

* stukken DNA die de erfelijke informatie bevatten voor de eigenschappen van een cel.
** een groot aantal kopieën.

Abbreviations

AMCA	amino-methyl-coumarinylacetic acid
bp	base-pairs
CARD	catalysed reporter deposition
CGH	comparative genomic hybridisation
CIS	carcinoma *in situ*
COBRA	combined binary ratio labeling
CT	combined tumor
DOP	degenerated oligoprimed PCR
DMs	double minutes
DNA	deoxyribonucleic acid
FISH	fluorescence *in situ* hybridisation
FITC	fluorescein isothiocyanaat
GCTI	germ cell tumors of neonates and infants
HSRs	homogenously staining regions
i(12p)	isochromosome 12p
Kb	kilobase
Mb	megabase
NS	nonseminoma
PCR	polymerase chain reaction
PLAP	placental alkaline phosphatase
PRINS	primed *in situ* PCR
SE	seminoma
SKY	spectral karyotyping
SS	spermatocytic seminoma
TE	teratoma
TGCT	testicular germ cell tumors of adolescents and young adults
YS(T)	yolk sac tumors

Dankwoord

"Voor het kennen van de weg, moeten we gewoon op weg. Je dingen doen, liefst met plezier, heel simpel op de Pooh manier"

Mijn weg begon in 1964 in Capelle a/d IJssel aan de Reigerlaan, als eerste uit het nest van Henk en Riet Mostert. Na het volgen van verschillende opleidingen kwam ik als groentje (roodje) terecht in het wetenschappelijk onderzoek bij TNO in Rijswijk. Daar werd mij al snel duidelijk dat wetenschap een echte hobby is. Dit werd vooral geïllustreerd door twee gedreven onderzoekers; Barb Trask en Ger v/d Engh. Hun interesse en enthousiasme voor zowel wetenschap als natuur hebben een belangrijke stempel gezet op het verdere verloop van mijn wegen, zowel wetenschappelijk als privé. Zij stimuleerden mij in het volgen van avondstudies en introduceerden mij in de meerdaagse "hikes". Ook was ik zeer blij met de mogelijkheid, jaren later in Seattle, een half jaar onderzoek bij hun te kunnen doen, en de gelegenheid die zij mij tijdens deze periode gaven om tevens wat werk voor LEPO te kunnen verrichten.

Een nieuw avontuur startte met Lambert Dorssers, die mij vroeg om als analist een lab te helpen opzetten op de Daniel den Hoed kliniek. Als "moleculair verpleegkundige" met de meest besproken benen van de Daniel, liet hij mij kennis maken met de echte moleculaire biologie en stelde mij bovendien in de gelegenheid om deeltijd biologie te gaan studeren in Utrecht. Ondanks onze goede band, bekoelde mijn interesse in het gesleutel aan het humane IL3-gen wat resulteerde in full-time studeren. De keuze om biologie te gaan studeren was een goede, al was het alleen al voor de ontmoeting met Reina. Reina, onze wegen komen voor een groot deel overeen (gelukkig voor Leendert niet helemaal) wat leidt tot vele gesprekken (tijdens hoogte en diepte-punten), wilde plannen en regelmatige uitstapjes (gaan we nu eindelijk kanoën?), kortom ik ben erg blij dat jij één van mijn paranimfen bent.

Droog brood op de plank dwong mij tot een bijbaantje in de diagnostiek bij LEPO. Na een half jaar werd er een nieuw project gestart en wisten Prof. Oosterhuis en Leendert Looijenga mij te motiveren voor hun onderzoek naar chromosomale afwijkingen in kiemceltumoren. Aangesteld op een analistenplaats gaven zij mij de mogelijkheid zelfstandig onderzoek te doen en ook gelegenheid om de resultaten op papier te zetten. Dat heeft uiteindelijk geleid tot dit proefschrift. Uiteraard is al dit werk niet alleen gedaan. Veel mensen hebben hieraan bijgedragen. Ondanks dat het waarschijnlijk leidt tot het onterecht vergeten van personen, wil ik toch een aantal mensen met name noemen. In eerste instantie Mirjam, oftewel "polleke". Nu ex-lid van het LEPO-team. Een groot deel van dit werk is ook jouw verdienste, ontelbare *in situ's* heb je gedaan en mede door jouw chromosomen-kennis konden we samen de CGH resultaten in Nijmegen analyseren. Samen met Annemieke Verkerk heb je ook sterk bijgedragen aan de mapping van het 12p-amplicon en binnenkort zal eindelijk blijken dat die buik niet alleen door het snoepen bij LEPO komt. Annemieke, jij hebt het 12p werk een flinke duw (in de goede richting) gegeven. Ondanks dat wij het duidelijk niet altijd met elkaar eens waren, deelden wij in elk geval de "Helleborus" interesse. Ook de overige (ex) LEPO-mensen (Jacqueline, Robert, Marjolein, Ruud, Ad, Hannie, Ton, Helene, Marja, Kirsten en Fons) hebben op de één of andere manier bijgedragen en ook wat verdragen. Met betrekking tot het laatste wil ik hierbij mijn oprechte excuses aanbieden voor mijn dagelijks terugkerende gezoem. Hans Stoop, één van de nieuwe LEPO aanwinsten, is met name zeer ondersteunend geweest bij de meer recente artikelen. Plezier in wetenschap wordt zeker door jou geïllustreerd en jij bent vast de volgende.

Het hier beschreven onderzoek is enkel mogelijk door het bestaan van de verzameling tumoren die het "LEPO-team" in de loop van de jaren heeft opgebouwd. Dit is gelukt door de bereidwilligheid van verschillende pathologen en urologen, dank hiervoor.

Prof. Oosterhuis; opperhoofd van de afdeling LEPO en tevens mijn promotor, u ben ik dankbaar om tenminste twee verschillende redenen. Ten eerste zorgde u, samen met Leendert, voor het bestaan van een project dat op mijn lijf was geschreven, maar vooral, voor het uit Groningen meenemen en binnen gezichtsveld brengen van "mijn" Leendert.

Het grootste deel van de CGH-analyses konden gelukkig dichter bij huis worden uitgevoerd, op de afdeling cytochemie en cytometrie (Leiden), met de hulp van Carla Rosenberg. This resulted in several publications but also a nice friendship. Since more than a year the common interest is expanded in baby-stuff. Carla, I really enjoyed the time in Leiden, thanks for all your support.....let's go to Bleiswijk.

Het afgelopen jaar is vooral gewijd aan het schrijven van dit boekje. Dit had als gevolg dat de sociale contacten op een laag pitje kwamen te staan. Dank aan iedereen voor het geduld en begrip, zullen we nu dan eindelijk die skate-, huis gaan bekijken-, thais eten- of zomaar afspraak maken??? Wat het schrijven ook zeer ten goede kwam was de bereidwilligheid van Gerard van den Aardweg om gedurende deze periode zijn kamer te delen met mij. Gerard, bedankt voor je zorgzaamheid en gastvrijheid.

Mijn aanwezigheid op LEPO werd ook gekenmerkt door tweemaal een periode van afwezigheid. Dit werd veroorzaakt door in eerste instantie de geboorte van Maarten, en 2,5 jaar later van Anna. Ik kon blijven werken omdat Maarten en Anna in goed handen waren/zijn. Halma bedankt voor je liefdevolle zorgen, jouw flexibiliteit en betrokkenheid is een onmisbare schakel in dit geheel.

De andere telgen uit het al genoemde Mostert nest zijn vervolgens; Mark, Saskia en Jeroen. Elk hebben ze een aandeel gehad. Mark, al zeg je weinig, leuk dat je regelmatig langskomt, iets wat speciaal door Anna wordt gewaardeerd. Het maakt niet uit dat jij soms voor vier eet, zodat we niks meer hebben om in te vriezen, en inderdaad; er zit al een glas om een flesje bier. Jeroen, niet enkel als bewijs dat erfelijkheid complex, maar het uiterlijke resultaat ten dele herhaalbaar is, ben ik blij met jou. Wij zijn het levende bewijs dat er vele wegen naar Rome leiden. Sas, we verschillen sterk op allerlei fronten, toch kunnen we heerlijk bomen en als wederzijdse praatpaal fungeren. Fijn dat je m'n zus en paranimf bent.

Als één van de laatsten maar zeker niet in de minste: pap en mam. Ook al staan jullie niet altijd te juichen bij mijn (weloverwogen) beslissingen, jullie altijd oprechte geïnteresseerdheid, medeleven, steun en bereidwilligheid om bij te springen als oma Riet en opa Henk, zijn van onschatbare waarde.

Lieve Leendert, dit boekje is eigenlijk het meest voor jou. Voor jou zal het ook een hele opluchting zijn als het allemaal achter de rug is. Geen werkbesprekingen meer in de auto en thuis..... we zullen het nog missen. Dank voor de ontelbare malen dat je de stukken hebt doorgelezen en gecorrigeerd, je hulp bij de figuren maken en vooral je (bijna) altijd positieve benadering en vertrouwen in mijn kunnen.....

Ik ga verder op weg, mijn dingen doen, liefst met plezier, heel simpel op de Marijke manier,

Marijke

Curriculum vitae

Marijke Mostert werd op 14 september 1964 geboren te Capelle a/d IJssel. In 1980 behaalde zij haar diploma aan de christelijke MAVO in De Lier. Hierna werd begonnen met de analistenopleiding aan het Van Leeuwenhoek Instituut te Delft waar de volgende specialismen met succes werden afgesloten: HBO-A botanie (1983), HBO-A zoölogie (1984), HBO-B biochemie (1987) en HLO biochemie (1988). Tijdens deze studies werden onderzoekstages uitgevoerd bij het Instituut voor Plantenziektekundig Onderzoek in Wageningen, het Radiobiologisch instituut TNO in Rijswijk en Lawrence Livermore laboratorium te Livermore in Californië (USA). In 1989 werd begonnen aan de studie biologie aan de universiteit in Utrecht welke in 1993 succesvol werd afgerond. Tijdens deze studie vonden twee onderzoeksstages plaats: 1) op de afdeling haematologie bij TNO in Rijswijk waar detectie van 5q deleties bij het myelodysplastisch syndroom werd geoptimaliseerd en 2) bij het Integraal Kanker Centrum in Rotterdam waar een voorlichtingsfolder voor beenmerg-donoren werd ontwikkeld.

Gedurende een deel van de studies heeft zij gewerkt als research analist op verschillende instituten: de afdeling flowcytometrie van het Radiobiologisch instituut TNO (1984-1987), de afdeling Moleculaire Biologie van de Dr. Daniel den Hoed kliniek (DDHK)(1987-1991) en het Laboratorium voor Experimentele Patho-Oncologie (LEPO/DDHK) (1993). Tevens was zij in 1993 freelance werkzaam als medewerkster voorlichting bij de DDHK. Vanaf januari 1994 t/m juni 1994 verrichte zij onderzoek op de afdeling Moleculaire Biotechnologie aan de Washington University in Seattle (USA). In augustus 1994 werd zij aangesteld als research analist op de afdeling LEPO (DDHK). Het verrichte onderzoek werd gefinancierd door de Nederlandse Kanker bestrijding (Koningin Wilhelmina Fonds, KWF) en staat beschreven in dit proefschrift. Marijke is getrouwd met Leendert Looijenga waarmee zij twee kinderen heeft; Maarten Wieger (1995) en Anna Marije (1998).

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Mostert, MMC, M van de Pol, D Olde Weghuis, RF Suijkerbuijk, A Geurts van Kessel, J van Echten-Arends, JW Oosterhuis, and LHJ Looijenga (1996). "Comparative genomic hybridisation of germ cell tumors of the adult testis; confirmation of karyotypic findings and identification of a 12p-amplicon." Cancer Genet Cytogenet 89:146-152.

Mostert, MMC, M van de Pol, J van Echten-Arends, D Olde Weghuis, A Geurts van Kessel, JW Oosterhuis, and LHJ Looijenga (1996). "Fluorescence in situ hybridisation-based approaches for the detection of 12p-overrepresentation, in particular i(12p), in cell lines of human testicular germ cell tumors of adults." Cancer Genet Cytogenet 87:95-102.

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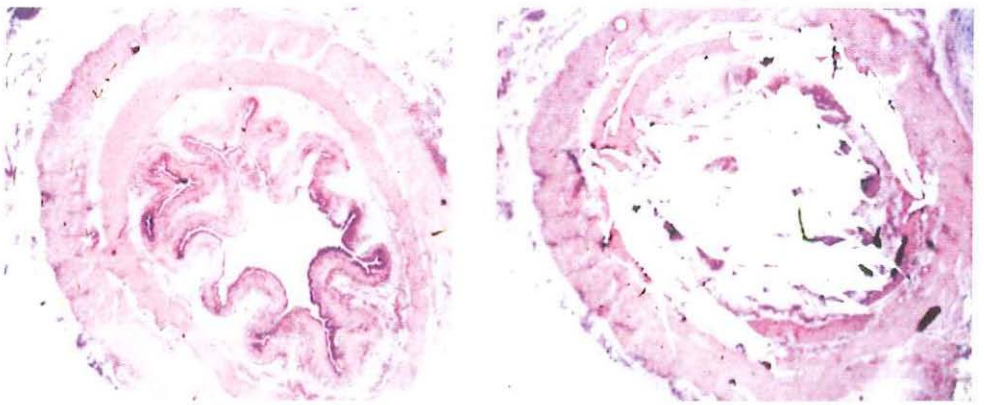
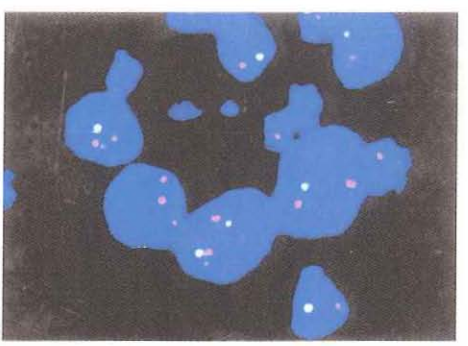
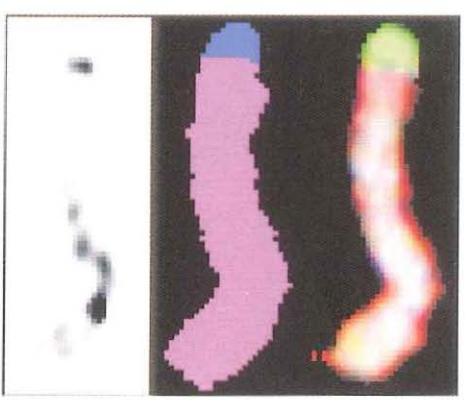


***Chapter 2. Fig. 1**: Representative example of a frozen tissue section of a teratoma of the infantile testis, before (left panel) and after (right panel) microdissection.*

***Chapter 2. Fig. 3**: Representative example of the double fluorescent in situ hybridisation with a probe specific for the centromeric region of chromosome 1 (red signal), and one for the 1p36 region (green signal) on a formalin-fixed, paraffin-embedded yolk sac tumor of the infantile testis (case no. 4). Note the reduced number of signals of the 1p36 region (green) compared to the centromere (red).*



***Chapter 7. Fig.2 B**: Representative example of banding (left panel) and spectral karyotyping (real color at the right and false color in the middle) of a chromosome 12 derivative, known to contain restricted 12p-amplification (as determined by in situ hybridisation and CGH). Note that the chromosome is composed of chromosome 12 material (indicated in purple), except the most distal region of the p-arm, showing chromosome 20-specific material (indicated in blue).*



Left page:

***Chapter 2. Fig. 4**: Representative examples of the immunohistochemical detection of the P53 protein (middle panel) and MDM2 protein (lower panel) on a tissue section of a formalin-fixed, paraffin-embedded teratoma (left panel) and yolk sac tumor (right panel) of the infantile testis. No P53 could be detected, while all tumor cells showed the presence of MDM2. The corresponding hematoxylin and eosin (H&E) stained slide is represented in the upper panel of the illustration. (magnification 100 X).*

Right page:

(upper)

***Chapter 3. Fig. 1:** Results of comparative genomic hybridisation of spermatocytic seminoma 2 (SS2) showing a representative example of a digital image of a normal metaphase spread (46,XY) hybridised with DNA isolated from the tumor (detected in green) and reference DNA (46,XY) (detected in red).*

(lower)

***Chapter 5. Fig. 3**: Example of the result of comparative genomic hybridisation on chromosome 12 of the seminoma component of combined tumor 2207, showing tumor specific amplification (represented as relative overrepresentation of the green compared to the red signal), encompassing region 12p11.1-p12.1 The blue line represents the ratio profile, i.e. the red to green fluorescence intensities over the whole chromosome length.*

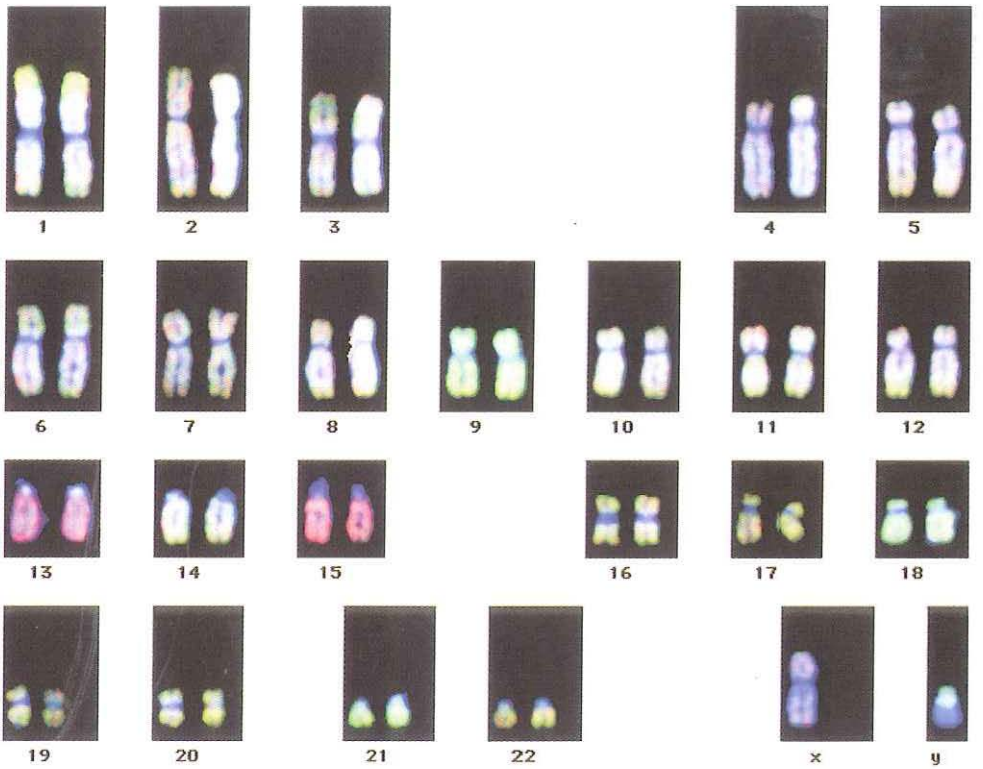
TERATOMA

YOLK SAC TUMOR

H & E

P53

MDM2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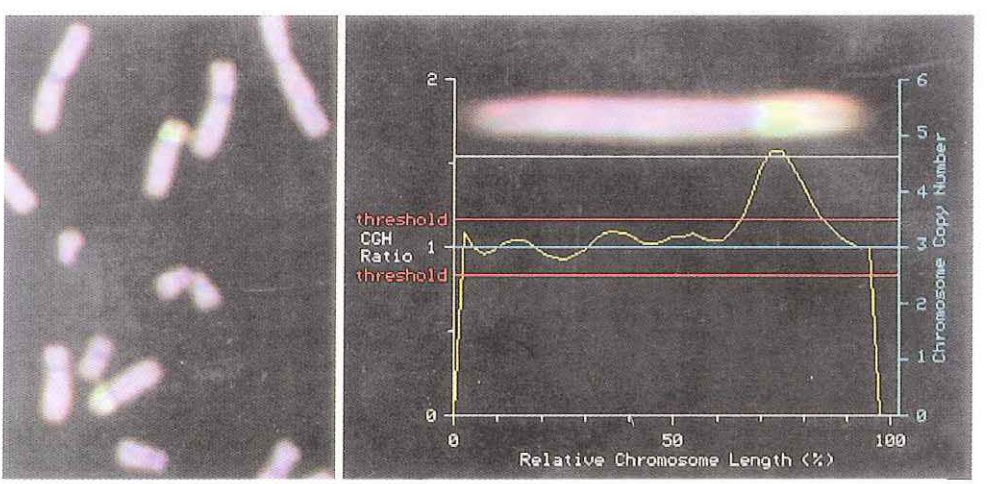
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Left page:

***Chapter 4. Fig.2**: (A) Double fluorescence in situ hybridisation (FISH) of a metaphase spread of S2 with the centromere-specific probe for pα12H8 (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 cytogenetically identified der(4)(4;12)(p13;q11) (lower right). (B) Double FISH with pα12H8 (TRITC) and yeast artificial chromosome (YAC#5) (FITC) on a metaphase spread of S2; there was no hybridisation of YAC#5 with the cytogenetically identified der(4)t(4;12)(p13;q11) (arrow). (C) FISH with pα12H8 (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 pα12H8 (FITC) and propidium iodide as counterstaining of the chromosomes showing enlarged centromeric hybridisation regions of the i(12p)s (arrows) as compared with their normal counterparts. (E) Original photograph of a double FISH with pα12H8 (TRITC) and YAC#5 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 hybridisation.*

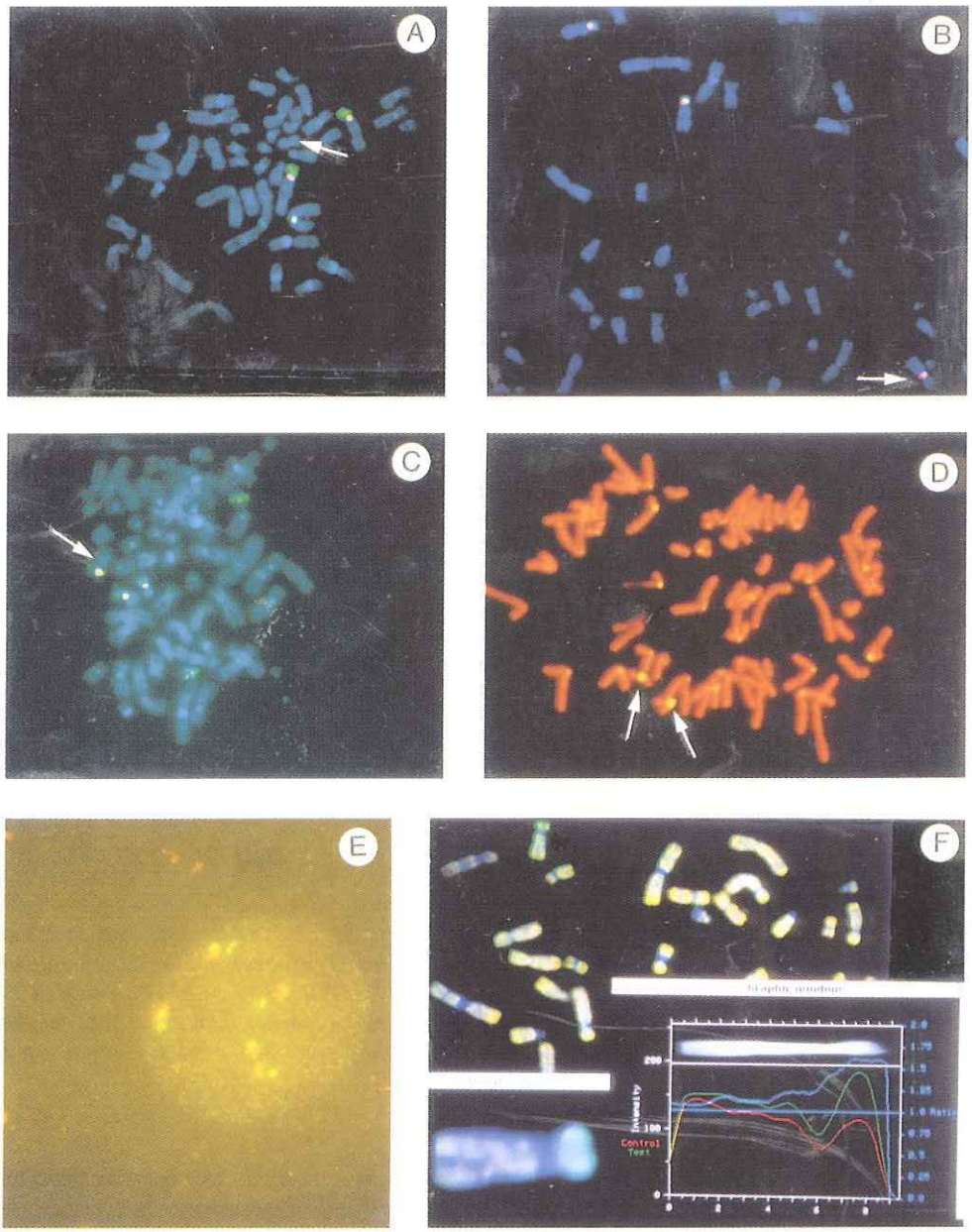
Right page:

(upper)

***Chapter 6. Fig. 2**: Example of bicolour fluorescence in situ hybridisation on isolated nuclei from tumor T8763, showing **(a)** similar signal numbers using YAC#5 (detected in green) and cosmid 31C2 (containing STS D12S1350E) (detected in red) as probes; and **(b)** a lower signal number for cosmid 124A3, specific for STS D12S1411 (detected in red) as compared to YAC#5 (detected in green). Signals for the cosmids (in red) are larger than the signals for the YACs (in green) because of a biotinyl-tyramide amplification step used to visualise the cosmids.*

(lower)

***Chapter 7. fig. 3**: Representative examples of double fluorescent in situ hybridisation on frozen tissue sections of 4 µm using a probe specific for the centromeric region of chromosome 12 (red) and YAC#5 (green), mapped within the shortest region of overlap of amplification (see Figure 1). Shown are A) carcinoma in situ; B) micro-invasive seminoma; C) invasive seminoma. The tumor cells are identified by the direct enzymatic alkaline phosphatase detection method (stained in red) on a parallel tissue section.*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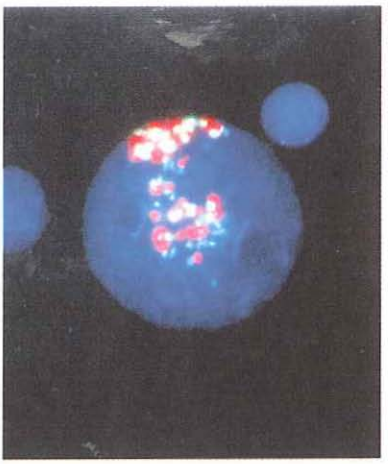

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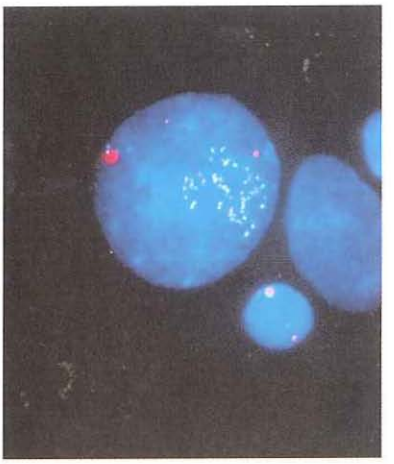

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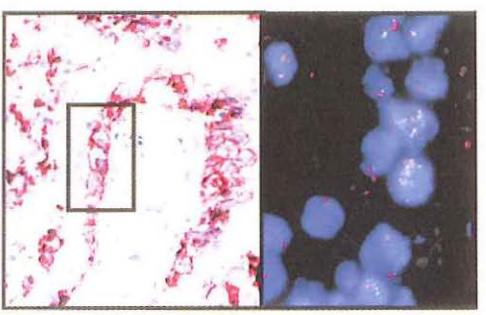

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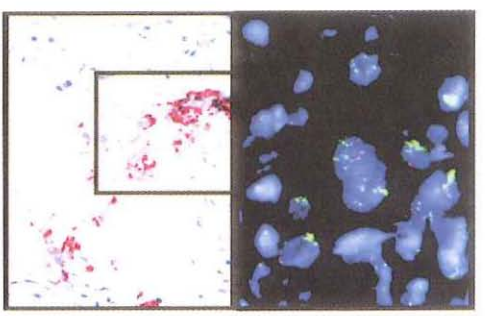

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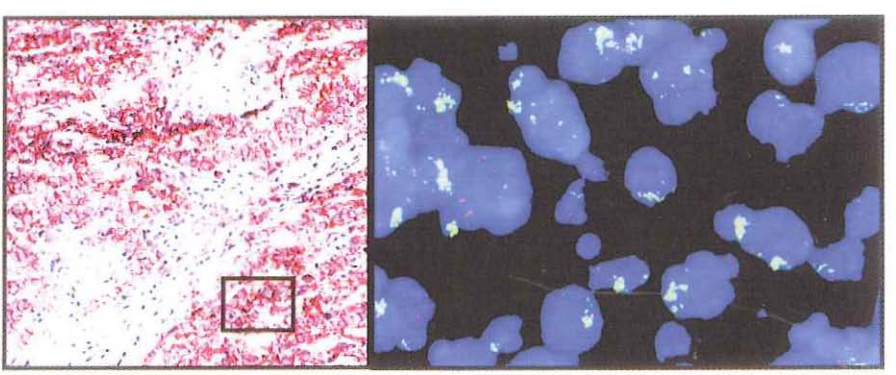

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