PATHOBIOLOGY OF GERM CELL TUMORS OF THE ADULT TESTIS;

Views and News.

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PATHOBIOLOGY OF GERM CELL TUMORS OF THE ADULT TESTIS;

Views and News.

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Kiemceltumoren
van de Volgroeide Teelbal;

Nieuwe Feiten en Ideeën.

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"Alles van waarde is weerloos"

Lucebert.

Aan Gerdi en Wieger Looijenga

CONTENTS

Chapter I. GENERAL INTRODUCTION. 9

1. Germ Cell Tumors of the Adult Testis: An Introduction. 10

2. Some Epidemiological Characteristics and Histological Classification. 10

3. Carcinoma In Situ of the Testis. 11

4. Genomic Aberrations in Germ Cell Tumors of the Adult Testis. 12

1. Introduction. 12
2. Ploidy. 13
3. Chromosomal Abnormalities. 13
A. Cytogenetic Analysis 13
B. In Situ Hybridization (ISH) 14
4. Molecular Abnormalities. 15
A. Tumor Suppressor Genes 16
B. (Proto-)Oncogenes 20

5. Genomic Imprinting, Methylation and Germ Cell Tumors of the Adult Testis. 21

6. Pathogenesis of Germ Cell Tumors of the Adult Testis. 23

7. Outline of the Thesis. 25

References: 26

Chapter II. Localization and polymorphism of a chromosome 12-specific α satellite DNA sequence. *Cytogenetics and Cell Genetics* 53:216-218, 1990. 37

Chapter III. *In situ* numeric analysis of centromeric regions of chromosomes 1, 12, and 15 of seminomas, nonseminomatous germ cell tumors, and carcinoma *In situ* of human testis. *Laboratory Investigation* 68:211-219, 1993. 43

Chapter IV. Clonality of combined testicular germ cell tumors of adults. *Laboratory Investigation* (in press). 55

Chapter V. Testicular germ cell tumors of adults show deletions of chromosomal bands 11p13 and 11p15.5, but no aberrations within the zinc-finger regions and exons 2 and 6 of the Wilms' tumor 1 gene. *Genes, Chromosomes & Cancer* 9:153-160, 1994. 63

Chapter VI. Involvement of chromosomes 12 and 22 in testicular germ cell tumors of adults. *Genes, Chromosomes and Cancer* (in press). 77

Chapter VII. Biallelic expression of the H19 and IGF2 genes in Human Testicular Germ Cell Tumors. *Journal of the National Cancer Institute* (in press). 93

Chapter VIII. Chromosome 15 allelic imbalances and parental methylation status in germ cell tumors of the adult testis. *Submitted.* 105

Chapter IX. Origin and biology of a testicular Wilms' tumor. *Genes, Chromosomes & Cancer* (in press). 119

Chapter X. GENERAL DISCUSSION. 133

1. Introduction. 134

2. Relationship between CIS, SE and NS. 134

3. Molecular Mechanisms causing Germ Cell Tumors of the Adult Testis. 135

4. Genomic Imprinting and Germ Cell Tumors of the Adult Testis. 140

5. Pathogenetic Model for Germ Cell Tumors of the Adult Testis. An Update. 141

Chapter XI. FUTURE RESEARCH. 145

1. Introduction. 145

2. Study of CIS. 145

3. Study of Genomic Imprinting. 147

Chapter XII. SUMMARY/SAMENVATTING 149

LIST OF PUBLICATIONS. 154

CURRICULUM VITAE. 156

DANKWOORD. 157

Chapter I.

GENERAL INTRODUCTION.

Paragraph 1.

GERM CELL TUMORS OF THE ADULT TESTIS: AN INTRODUCTION.

Germ cell tumors are on the crossroads of cancer and developmental biology. Recent progress in the understanding of genomic imprinting for example has re-emphasized the value that these malignancies may have for the study of human early embryogenesis. This thesis deals with testicular germ cell tumors of adolescents and adult men (TGCTs). Because of its different pathogenesis, spermatocytic seminoma is not included (1-4).

In the first chapter the literature on TGCTs is reviewed with emphasis on histology (paragraph 2), the precursor lesion (paragraph 3), and the genomic aberrations (paragraph 4). In addition, in paragraph 5 TGCTs are discussed from a developmental biology point of view, with genomic imprinting as major theme. In paragraph 6 the different pathogenetic models for TGCTs are summarized. Finally (paragraph 7) the aims of this thesis are presented.

Paragraph 2.

SOME EPIDEMIOLOGICAL CHARACTERISTICS AND HISTOLOGICAL CLASSIFICATION.

The highest incidence of TGCTs is in men aged 15 to 45 years (5, for review). Although rare (1-3% of all malignancies in white males), it is the most common cancer in this life period, comprising approximately one third of all malignancies. The incidence has more than tripled since 1940, and is still increasing (6,7, for review).

All TGCTs originate from germ cells (8). They have a fascinating histology, showing striking similarities with tissues found during early embryogenesis (also I.5). Histologically TGCTs are typed according to the WHO classification (9). Two, clinically relevant, categories can be distinguished; seminomas (SE) and nonseminomatous TGCTs (NS). SE are composed of solid fields of uniform cells with ample, clear cytoplasm and distinct cell borders. The solid areas are separated by connective tissue septa with varying numbers of lymphocytes. This histology is illustrated in Figure 1A.

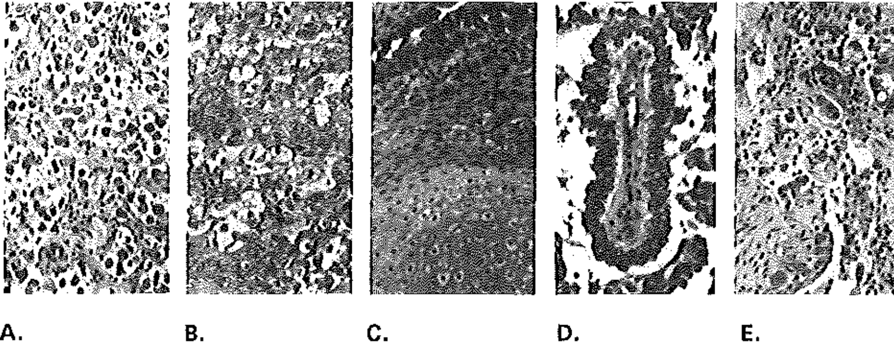

Fig. 1. Histology of the different types of testicular germ cell tumors of adolescents and adults, showing (A) seminoma, (B) embryonal carcinoma, (C) teratoma, (D) yolk sac tumor, and (E) choriocarcinoma. (Hematoxylin-eosin staining, 100X)

The highest incidence for SE is in the fourth decade of life (5,10,11). In contrast to SE, NS is histologically very heterogeneous. It can be composed of embryonal carcinoma (EC); teratoma (TE) (immature (I) and/or mature (M)); yolk sac tumor (YS); and choriocarcinoma (CH) (9). Most NS have a mixed histology, with different elements present in various proportions (7, for review). These different histological elements may be separated geographically, or truely mixed. Representative pictures of the histologies are shown in Figure 1B-E. NS are most frequent in the third decade of life (5,10,11).

Of the group of TGCTs, SE and NS constitute 50% and 40%, respectively (7,12). The remaining TGCTs are composed of both a SE and a NS component, classified as NS according to the WHO classification (9), and as combined tumor according to the British classification (13). We will use the term combined tumor (CT). The mean age of clinical presentation of CT is in between that of pure SE and NS (10,11).

The increasing incidence of TGCTs affects both SE and NS (5,12, for review). This suggests that their pathogenesis is related, and that they may originate from a common precursor, which will be the subject of the next paragraph.

Paragraph 3.

Carcinoma In Situ of the Testis.

The first report of abnormal cells in the seminiferous tubules adjacent to a TGCT was made in the late 19th century (14). In spite of the description of these intratubular abnormal cells as "an early stage of seminoma" (15), the pathogenetic relationship between these carcinoma in situ cells (CIS) and TGCTs was only firmly established in the early seventies. At that time Skakkebæk (16) reported two infertile men with CIS in their testicular biopsy who subsequently developed an EC. The relationship between CIS and TGCTs is also demonstrated by the presence of CIS in the parenchyma of most testes bearing a SE, a NS or a CT (17-19). Besides CIS, other terms are being used, e.g., "intratubular germ cell neoplasia, unclassified type" (IGCNU), "intratubular germ cell

neoplasia" (IGCN), "testicular intra-epithelial neoplasia" (TIN), and "uncommited malignant germ cell" (UMGC). We prefer "gonocytoma in situ" (GIS), but we shall use the term CIS, since it has gained broad acceptance, although it is strictly speaking not correct.

Thus far, apart from their anatomical localization no differences have been reported between CIS- and SE cells (also I.4.2 and I.4.3). The typical histology of CIS is shown in Figure 2.

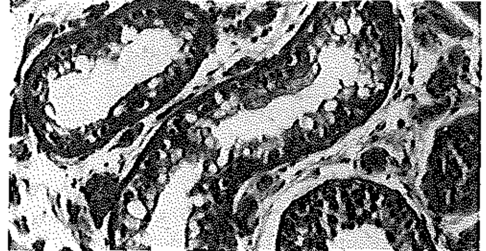

Fig.2.
Representative example of the histology of carcinoma in situ of the testis.
(Hematoxylin-eosin staining, 100X)

Immunohistochemically germ cell specific alkaline phosphatase (detected by anti-placental-alkaline phosphatase antibodies), 43-9F and tra-1-60 expression has been found in CIS as well as in SE (20-24). In addition, they both show morphological and ultrastructural similarities with early (primordial) germ cells (25-27).

Based on the similarity of the accumulated life time risk of TGCTs and the sum of the incidence of CIS and TGCTs in Danish men (28), it has been concluded that CIS will always progress into an invasive TGCT, and does not spontaneously regress. Follow up data of men diagnosed for CIS without an invasive TGCT support this hypothesis (19,29-31). Therefore, the presence of CIS alone, is sufficient to justify the diagnosis of a TGCT.

Paragraph 4.

GENOMIC ABERRATIONS IN GERM CELL TUMORS OF THE ADULT TESTIS.

1. Introduction.

After the finding in the beginning of this century that cancer is associated with somatic chromosomal aberrations (32), clonal evolution from one single precursor cell was hypothesed. This "stem cell theory" was subsequently demonstrated to be true for a number of cancers using cytogenetics, molecular and biochemical approaches. In the course of the evolution of cancer, the malignant cells will loose and acquire characteristics, resulting in divergence from their parent cell(s) (33).

A variety of somatic genomic aberrations has been reported in cancer (reviewed in 33,34), some of them more or less specific for a particular type of cancer. In summary, the following types can be distinguished: loss and/or gain of whole chromosomes; loss

and or gain of specific chromosomal regions; structural chromosomal abnormalities, like translocations and inversions; other kinds of aberrations, like point mutations. In general all these aberrations result in disturbance of the function of one or more genes. In most (solid) cancers more than one of these types play a role, either simultaneously or in a sequential order. This implies a multistep process towards the malignant phenotype, well documented in the adenoma-carcinoma sequence in colon (35, for review).

Genetic factors, that might be transmitted through the germ line, are also involved in the pathogenesis of TGCTs, suggested by many data, e.g., racial differences in incidence of TGCTs (highest in Denmark, lowest in Nigeria), even in Europe there is a remarkable geographic variation (highest incidence in Denmark, lowest incidence in Finland (5)), familial clustering of TGCTs (36, for review) and correlation with HLA subtypes (36-38, for review).

Thus far, the role of the different mechanisms in the pathogenesis of TGCTs has mainly been studied in the invasive part of the cancer. The results are summarized in the next three paragraphs.

2. Ploidy.

Due to loss and/or gain of (parts of) chromosomes cancer cells may develop an aneuploid DNA content during progression (39). This can be studied by flow cytometry (FCM) or image analysis (IA). In contrast to FCM, IA may identify minor aneuploid subpopulations as well as diploid and (near) tetraploid malignant cells, but it gives a lower stem line resolution (40,41).

Ploidy measurements using FCM and IA show that TGCTs are virtually always aneuploid (10,42-46). Even the relatively benign, well differentiated cells of residual mature teratoma are aneuploid (47,48). DNA content analysis of CIS (before and after progression into an invasive cancer) shows a DNA content between hypertriploid and hypopentaploid (16,45,49-51). The largest study by De Graaff et al., using FCM of CIS adjacent to 14 SE, 25 NS and 16 CT, shows a hypertriploid DNA content.

SE and NS (pure or mixed) have a significantly different ploidy: hypertriploid and hypotriploid, respectively (10,44,45). No differences in ploidy between classical SE and anaplastic SE have been found (52). Anaplastic seminoma reportedly has a higher fraction of cells in S-phase than classical seminoma.

In conclusion; invasive TGCTs, as well as CIS, are characterized by a near-triploid DNA content. CIS and SE are usually hypertriploid and NS is usually hypotriploid.

3. Chromosomal Abnormalities.

A. Cytogenetic Analysis

Detection of cancer-specific chromosomal abnormalities became feasible with chromosome banding techniques, developed in the early seventies (53, for review). This approach was also valuable for identification of mechanisms involved in the pathogenesis of TGCTs.

The consistent aneuploidy of TGCTs detected by FCM and IA (I.4.2) was confirmed using cytogenetic analyses (54,55, for review). De Jong et al., applied direct

harvesting of metaphases to SE and short term culture to NS. They found a striking pattern of over- and underrepresentation of chromosomes, which was highly similar for SE and NS. The chromosomes 7, 8, 12, and X were over- and the chromosomes 11, 13, 18 and Y were underrepresented. In addition, they found that the chromosomes 15 and 22 had higher copy numbers in SE than in NS, partially explaining the higher DNA content of SE compared to NS (I.4.2). In their much smaller and less homogeneous material of SE and NS karyotyped after short term in vitro culture, Rodriquez et al., (55) did not find this non-random pattern of over- and underrepresentation of chromosomes.

The most consistent structural chromosomal aberration in TGCTs is the isochromosome 12p (i(12p)), first described by Atkin and Baker in 1982 (56), and subsequently frequently confirmed by others (55,57-62). Most TGCTs, both SE and NS, pure or mixed, contain one or more copies of i(12p). Rearrangements of chromosomal band 12p13 were reported in 35% of i(12p) negative TGCTs, and in only 3% of TGCTs containing an i(12p) (63). Apart from those involving 12p other structural chromosomal abnormalities have been described, some with possible clinical relevance (chromosome 1 rearrangements and 1p deletions (58)), some supposedly correlated with histological type (rearrangement of chromosome 1p32-36 (55,59) and 7q11.2 correlated with TE, and 1p22 with YS (55)). One study reports deletions of the long arm of chromosome 12, region q13-q22, in 44% of pure or mixed NS (62). Deletions affecting region q11-q13 are only found in post-therapy samples. Subsequently, clusters of breakpoints have been reported in the chromosomal regions 12q11, q13, q15, q21, q22, q24, and q25 (55). In contrast, no breakpoint clustering was found in a series of 18 primary testicular SE, 50 primary testicular NS and 27 residual mature teratomas (Van Echten, submitted). This study reveals i(12p) as the only consistent structural chromosomal abnormality for TGCTs.

The role of the sex chromosomes in the development of TGCTs is still controversial. The presence of X chromatin, referred to as "female-pattern", is reported in some testicular TE and EC, but not in SE (64, for review). Excess of X compared to Y, probably due to loss of Y chromosomes, has been demonstrated cytogenetically in SE and NS (54,65, for review), which is supported by molecular analyses (66).

Different histological components within a NS, even with major differences in malignant behaviour, may have a highly similar genomic constitution (67,68). Chromosomal analysis of the SE and NS component of CTs shows common structural abnormalities in most of the cases (69-71).

Few data are available about the chromosomal constitution of CIS. Vos et al. reported three cases in which direct harvesting of parenchyma adjacent to the invasive TGCT was successful (72). One contained an i(12p), while the other two lacked it. In one of the latter two cases the invasive part of the tumor lacked the i(12p) as well. In all three cases the chromosomal constitution of CIS and the invasive component was different.

B. In Situ Hybridization (ISH)

Cytogenetic analysis has a number of drawbacks. It needs direct harvesting or short term culture, which is often not possible. Tissue culture may introduce biases due to selection of cells with growth advantage in vitro. Cytogenetic analysis is not possible on frozen or paraffin embedded archival tissue. A recently developed technique, known as DNA *in situ* hybridization (ISH), is a useful adjunct to cytogenetic analysis. The technique

uses probes specific for certain (parts of) chromosomes (73). Based on the homology of the target and a labeled probe, an overall estimation of the number of targets can be made. The technique is schematically illustrated in Figure 3.

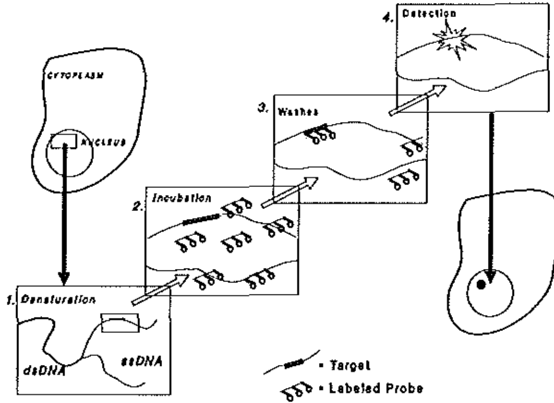

Fig. 3. Schematic representation of the DNA In Situ hybridization technique.

Essentially this approach has two applications. It can be used to extend the morphological analysis of chromosome spreads, and as an entirely new modality it allows interphase cytogenetics (73-76, for review). Using ISH, the constitution of the morphologically defined i(12p) has been confirmed (77,78). In addition, overrepresentation of 12p material, hidden in other chromosomal rearrangements than i(12p), has been reported in all "i(12p)-negative" TGCTs thus far (78,79). ISH on tissue sections allows numeric analysis of specified subpopulations of cells within their histological context (80-82). Using this approach, aberrant numbers of centromeric regions of chromosome 1 in interphase nuclei of CIS as well as of NS have been reported (83,84). In addition, CIS cells have been identified in semen using this technique (85).

The chromosomal constitution of CIS and its adjacent TGCT have not been compared systematically. Questions concerning the presence of i(12p) in CIS, the karyotypical relationship between different histological invasive components, as well as between CIS and its adjacent invasive component, can be answered using ISH. Results of these studies will deepen our understanding of the pathogenetic relationship between CIS, SE and NS.

4. Molecular Abnormalities.

The most fundamental approach to the study of cancer is the elucidation of the underlying molecular genetic mechanisms. Cytogenetics plays an important role since it has in many instances by demonstrating consistent structural abnormalities indicated which chromosomal regions were of interest for molecular studies. However, point mutations, small deletions and amplifications can be missed by cytogenetic analysis.

In principle two types of genes are involved in malignant transformation: the proto-

oncogenes and the tumor suppressor genes (33). Both classes of genes are important in the regulation of normal proliferation and differentiation. The proto-oncogenes can be activated, due to point mutation(s), small insertion(s), deletion(s) or translocation(s), to become oncogenes which act in a dominant manner (86-88, for review). In contrast, the tumor suppressor genes may act in a recessive or dominant manner. Involvement of tumor suppressor genes usually requires loss of function (89-91, for review). The available data about the role of these genes in the development of TGCT are summarized in the next two sections.

A. Tumor Suppressor Genes

Indicative for the loss of tumor suppressor gene activity in the development of cancer is the presence of consistent deletions. These may be detectable by cytogenetics when relatively large chromosomal regions are involved, otherwise they may be identified by screening for loss of heterozygosity. Cytogenetic analysis suggests the involvement in the pathogenesis of TGCT of tumor suppressor genes, localized on chromosomes 1 and 11 (92). In addition, specific underrepresentation of (parts of) chromosomes (I.4.3.) may also indicate that loss of tumor suppressor gene activity is involved. The presently known tumor suppressor genes, and their chromosomal localization and their involvement in cancer development (91,93,94), are summarized in Table 1. The chromosomal regions assumed to contain a tumor suppressor gene are also illustrated.

The results of screening for loss of heterozygosity (LOH) in TGCTs with conventional techniques are summarized in Table 2. These data illustrate the heterogeneity in histologically similar components, and the varying involvement of (parts of) chromosomes, even when studied with the same probes. Not all chromosomes have been intensively studied for the presence of aberrations, e.g., chromosomes 4, 8, 9, 10, 14, 15, 16, 18, 20, 21, and 22 are less studied than the others. Some tumor suppressor genes have been studied in more detail in TGCTs.

The Retinoblastoma (*RB*) gene is only expressed (analyzed by Western and Nothern blotting and immunohistochemistry) in testicular TE (M), without gross genomic alterations (95). Immunohistochemistry shows *P53* expression in TGCTs of all histological types, including CIS, with a more intense staining in EC compared to SE (96). No mutations in the coding regions of this gene were found in a series of 22 TGCTs and in the germ line of 17 members of families with an increased risk for the development of this cancer (97). In contrast, mutations were found in four out of 17 primary SE of chinese patients, affecting exons 5, 7 or 8 (98).

None of the chromosomal regions containing a (putative) tumor suppressor gene is consistently involved in TGCTs, except the short arm of chromosome 11, bands p13 and p15.5. From the 11p13 region the tumor suppressor gene *WT1* was isolated (99-101). In familial and sporadic Wilms' tumors the function of this gene is disrupted by mutations or deletions (102-107), as well as in Denys Drash and WAGR syndrome patients (108-115), both syndromes carry an enhanced risk for renal Wilms' tumors. Thus far only approximately 10% of the sporadic renal Wilms' tumors contain a mutation in the WT1 gene, usually in one of the zinc-finger regions (116-118). Therefore aberrations due to deletions or mutations affecting these regions of this gene seem not to be crucial for the development of sporadic Wilms' tumors.

Table 1.
Overview of the isolated and putative*
tumor suppressor genes, their chromosomal localization and associated disease.

Chromosome	Region	Gene	Malignancy/ Syndrome
1		?	melanoma
	p36	*NB*1*	neuroblastoma
	p	?	breast cancer
			neurofibroma
			meningioma
	q	?	breast cancer
3	p	?	small cell lung cancer
	p14	*RCC**	renal cell carcinoma
	p25	*VHL*	pheochromocytomas carcinomas colon and kidney (Von Hippel-Lindau)
	q	?	renal cell carcinoma
5	q21	*APC,MCC*	colorectal cancer
6	q	?	melanoma
7		?	melanoma
9	p	?	astrocytoma
	p21	*MTS*1 (p16)	melanoma
			glioma
			osteosarcoma
			astocytomas
			carcinomas: bladder, breast, lung, ovary, kidney
	q31	*BCNS**	medulloblastoma
			skin cancer
10		?	astrocytoma
			melanoma
11	p	?	breast cancer
	p13	*WT*1	Wilms' tumor
			Denys Drash, WAGR
	p15	*WT2*,H19*	Beckwith-Wiedemann
	q13	*MEN*1*	pituitary adenoma
13	q	?	breast cancer
	q14	*Rb*	retinoblastoma
			breast cancer
			small cell lung cancer
			sarcoma
14	q	?	neurofibroma
			neuroblastoma
			meningioma
17		?	neurofibroma
			neuroblastoma
			meningioma
	p13	*P53*	glioblastoma multiform
			carcinomas (e.g., breast)
			small cell lung cancer
			colorectal cancer
			(osteo)sarcomas
	q11	*NF*1	neurofibromatosis type 1
			glioma
	q21	*BCRA*1*	carcinoma breast, ovary
18	q21	*DCC*	colorectal cancer
19		?	melanoma
22		?	meningioma
	q22	*NF2*	neuro fibromatosis type 2

* Not cloned yet.

Table 2. Summary of allelic imbalances in Testicular Germ Cell Tumors.

Chromosomal region	Locus	Probe	Abnormalities (%) [numbers studied] SE	NS	CT	Reference(s)
			[6	5	3]	Radice et al., 1989
1p	NGFB		17			
1p32	MYCL	l-myc				
			[6	4	1]	Peltomäki et al., 1990
1p36	D1Z2	p1-79				
1	D1S51	CRI-L1191				
1	D1S71	CRI-L1039				
			[19	12]		Lothe et al., 1989
1p	D1S57	YNZ2				
2pter-q32	D2S44	YNH24				
			[6	5		Radice et al., 1989
2p24	MYCN	pMY820				
3p21	DFN15S2	pHM2.6				
			[19	12]		Lothe et al., 1989
3p	D3S32	EFD145 :	37	9		
3p21-p14	D3S2	pHF12-32 :				
			[6	4	1]	Peltomäki et al., 1990
3p21	DNF15S2	pH3H2				
5p	D5S69	CRI-L1072	33	25		
5q12-q14	D5S39	105-153A :				
5q21	D5S37	Pi227 :	33	75		
5q21-q22	D5S4	pMC5.61 :				
			[6	5	3]	Radice et al., 1989
5q21-q22	D5S4	L1.4				
6p21.3	HLA-DQA	pDCH1				
	DXA					
6q22-q23	MYB	pHM2.6				
			[6	4	1]	Peltomäki et al., 1990
7p	D7S62	CRI-L1020	33	25		
7q21	PGY3	pMDR2 :				
7q21.3-q22.1	COL1A2	pNJ3/3.5 :				
7q31-q32	MET	pmetH :	33	50		
7q22-q32	D7S8	pJ3.11 :				
7	D7S59	CRI-L887 :				
			[6	5	3]	Radice et al., 1989
7q	TRCB	pJ2				
7q31	MET	pmetD				
			[19	12]		Lothe et al., 1989
8	D8S18	YNZ132				
			[6	4	1]	Peltomäki et al., 1990
9q34	ABL	abl-K2				
			[6	5	3]	Radice et al., 1989
10q11.2	PTC	p1.2			33	
			[6	4	1]	Peltomäki et al., 1990
11p15.5	HRAS1	pT24-C3				
11p15.5	INS	pHINS310				
11	D11S134	L834				
			[6	5	3]	Radice et al., 1989
11p15.5	HRAS1	pbc-N1				
11p15.5	INS	pHINS310				
11p15.5	pADJ762	D11S12				
			[19	12]		Lothe et al., 1989
11p15.5	HRAS1	pTBB-2 :	16	17		
	INS	phins310:				
			[19	12]		Lothe et al., 1993
11p15.4	CALCA					
	PTH	p20.36				

			[6		5	3]	Radice et al., 1989
11p15.5	PTH	p20.36	17		40	33	
11p13	CAT	PCATint800					
			[19		12]		Lothe et al., 1993
11p13	FSHB						
	D11S16	P32-1 :	42		33		
	D11S325	:					
11q13	PGA	PGA101 :					
			[6		5	3]	Radice et al., 1989
11q13	INT2	SS6					
11q23	ETS1	pHE5.4					
			[6		4	1]	Peltomäki et al., 1990
12p13.2	PRB1	pPRP112.2RP					
12q14.3	COL2A1	pPST1 :					
12q14.3	D12S7	pDL32B :					
12	D12S28	CRIU-L375:	17				
12	D12S27	CRI-L409 :					
			[6		5	3]	Radice et al., 1989
12q14.3	D12S7	pDL32B					
			[4		41]		Murty et al., 1992
12q12-13.1	D12S15	pCMM1.2 :					
12q13.1	D12S17	pYNH15 :					
12q13	D12S14	pEFD33.2:					
	D12S4	p9F11 :		>40			
12q13.2-13.3	D12S6	11-1-7 :					
12q14-15	D12S8	p7g11 :					
12q22	D12S7	pDL32B :		>40			
	D12S12	pAC230 :					
	MGF	pHKITL					
12q23	IGF1	phigf1					
12q21-22	IAPP	CRIAP8					
12q13-q14	INT1						
	GLI1						
	MDM2						
			[1		2 (and 2 cell lines)]		Geurt van Kessel et al., 1989
			[19		12]		Lothe et al., 1989
13q12-q12	D13S1	p7F12					
			[6		5	3]	Radice et al., 1989
13q12-12	D13S1	p7F12					
13q33-qter	D13S3	p9A7			20		
			[6		4	1]	Peltomäki et al., 1990
13q12-q14	D13S1	p7F12					
13q22-q31	D13S4	Pp1E8					
			[6		5	1]	Radice et al., 1989
16q22	HP	Hp2α					
			[6		4	1]	Peltomäki et al., 1990
16q22-q24	D16S7	p79-2-23					
17p	D17S34	p144D6					
			[19		22]		Lothe et al., 1989
17p	D17S30	YNZ22	[6		4	1]	Peltomäki et al., 1990
18	D18S16	CRI-1261	[6		5	3]	Radice et al., 1989
19p13	INSR	pHIR/P12.1					
			[19		22]		Lothe et al., 1989
19	D19S20	JCZ3.1					
			[6		4	1]	Peltomäki et al., 1990
19q13.2-cen	D19S11	p13-1-25					
21q22.1	SOD1	SOD1					
22q11	BCR	bcr Pr-1					
			[6		5	3]	Radice et al., 1989
22q12-13	PDGFB	pv-sis					

It might be worthwhile to study the chromosomal regions and genes involved in Wilms' tumors also in TGCTs for the following reasons: chromosome 11 is underrepresented in TGCTs (I.4.3); approximately one in three TGCTs contains a deletion of (a part of) the short arm of chromosome 11 (I.4.4.A); extrarenal Wilms' tumors may be derived from germ cells (124-126), and TGCTs may have Wilms' tumor as one of its components (unpublished results).

B. (Proto-)Oncogenes

Overrepresentation of certain (parts) of chromosomes in TGCTs might indicate the involvement of oncogenes (I.4.3). Some candidate genes have been studied in TGCTs on the DNA, RNA and/or protein level. The p62[myc]-oncogene product is present at higher levels in SE than in different components of NS (127). An enhanced N-*MYC* expression from a single gene, correlated with a diminished Rb expression, was reported in one SE (128). Expression of N-*MYC* without gene amplification was also detected in primary SE and EC, while no expression was found in primary TE components. This study also illustrates that L-*MYC* is not expressed (129).

Of special interest in TGCTs is the short arm of chromosome 12 (I.4.3). The consistent overrepresentation of 12p material in TGCTs indicates its importance in their pathogenesis. The Ki-*RAS* gene was studied as a candidate gene. Amplification and enhanced expression of Ki-*RAS* was detected in EC cell-lines (TERA 1 and 2), and in more than 50% of EC metastases (130). This is in agreement with a Ki-*RAS* gene dosage increase in two other series: 78% and 50%, respectively (62,131). No mutations were found in the Ki-*RAS* gene in seven i(12p) positive NS cell lines, in spite of an enhanced expression (132). This is in agreement with another study of nine NS cell lines, including two lines also studied in the series of Dmitrovsky (133). In contrast, 40% of 14 primary testicular SE have been reported to contain mutations in the Ki-*RAS* or N-*RAS* (mapped to the short arm of chromosome 1 (134)) in a heterogeneous pattern within the cancer (135). No mutations were found in Ha-*RAS* or Ki-*RAS* in a series of 31 TGCTs, comprising 22 SE and 9 NS (136), while this study reports that 59% of the SE and 56% of the NS contain N-*RAS* mutations at codon 12 and/or 61. Recently 6% of 18 primary testicular SE and 12% of 25 primary testicular NS were shown to have Ki-*RAS* mutations at codon 12 (137). A series of 17 primary TGCTs, 11 SE and six NS, showed Ki-*RAS* mutation at codon 12 in two SE, and no mutations in the N-*RAS* gene (138). *PTHRP* was found to be expressed in all SE, and CH components, but not in EC and TE (139).

In TGCTs a gene dosage increase for *PDGFA* was found, without enhanced expression (131). This study showed no consistent alterations in N-*RAS*, *MYC*, *KIT* (stem cell factor (SCF) receptor), *MYB*, *ABL*, Ha-*RAS*, *P53*, and *ERBB*2 on either DNA or RNA level. In addition, expression of *HST*1 is found in TGCTs without obvious differences in gene dosage, while *INT*2 is not expressed (139,140). A modulated expression of *HST*1 is also reported in a EC cell line, as well as in primary TGCTs (141). These results are partly supported by a study of 70 (142) and 24 TGCTs (139), revealing *HST*1 expression in the majority of NS and in minority of SE. One study (142) shows expression of *KIT* in 80% of SE and 7% of NS. Using three NS cell lines (NT2D1, N2102EP and 240A), as well as primary TGCTs (10 SE, 12 NS and 2 CT), an other study (122) reports expression for *SCF* and *KIT*. Three percent of the SE and 50% of the NS show *SCF* expression, while 30% of the SE and none of the NS express *KIT*.

Paragraph 5.

GENOMIC IMPRINTING, METHYLATION AND GERM CELL TUMORS OF THE ADULT TESTIS.

The gamut of different embryonal and extraembryonal tissues present in TGCTs (I.2) suggests that mechanisms involved in regulation of early embryonic development are involved in its pathogenesis. During the eighties a new phenomenon important in early embryonal development has been discovered, not obeying the Mendelian rules of inheritance. Pronuclear transfer experiments in mice illustrated that the developmental potential of a manipulated diploid zygote was determined by the parental origin(s) of the haploid sets of chromosomes present (143). Uniparental embryos always thwart early in pregnancy. They rarely survive beyond day ten. Androgenotes, zygotes with an exclusively paternal genome, show a poor development of somatic tissues, but the extraembryonic tissues, in particular the trophoblast develop relatively well. In contrast, the gynogenotes, zygotes with a completely maternally derived genome have a relatively normal development of somatic tissues and a very poor growth of the trophoblast (144, for review). Apparently for normal embryonic development certain chromosomal regions must be donated by the father and some by the mother (144,145, for review). This difference in functionality between homologous chromosomal regions based on their parental origin is defined as genomic imprinting (GI) (143). Somewhere during the development of the primordial germ cell to the mature gamete, the genomic constitution of both paternally and maternally imprinted chromosomes, present in the normal zygote, has to be changed into either a totally maternal pattern (during oogenesis), or into a totally paternal pattern (during spermatogenesis) (146, for review). This is schematically illustrated in Figure 4. It is somewhere along this route, that TGCTs are initiated (I.6).

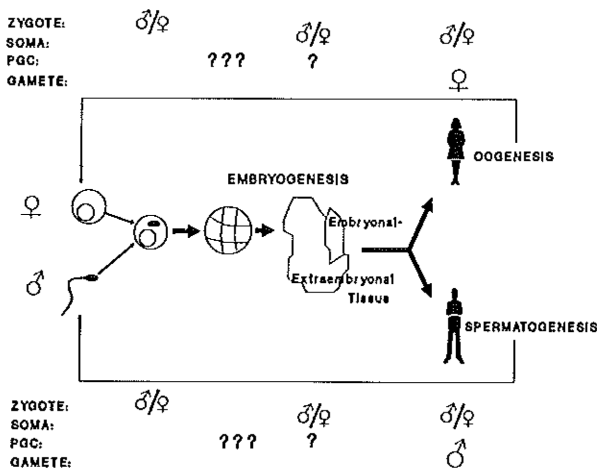

Fig. 4. Schematic representation of the processes of erasement and imprinting in the germ cell lineage in mammals.

A role of GI is suggested for a growing list of human pathological conditions (147-149, for review). A recent analysis of the parental origin of the extra set of chromosomes in triploid fetuses illustrates their similarity with murine androgenotes and gynogenotes (150). A preferential involvement of one of the parental chromosomal regions in cancer is interpreted as an indication that GI is involved. For example, renal Wilms' tumors are characterized by exclusive loss of the maternal and retention of the paternal 15.5 band on the short arm of chromosome 11 (151). In contrast, TGCT show preferential loss of the paternal 11p region and retention of the maternal region (121). However, the overrepresented short arm of chromosome 12 in TGCTs may have paternal or maternal origin (152), but is always from the same parent within one cancer (153).

Several murine genes have been reported thus far to show a uniparental pattern of expression during normal development. The *H19* and *igf2*-receptor genes are only expressed from the maternal allele (154,155), while *igf2* and *Snrpn* are expressed from the paternal allele (156-158). In addition, allele specific expression in the yolk sac is recently reported for the *ins1* and *ins2* genes (159). The human homologs of *H19*, *igf2* and *Snrpn* have the same imprinting as in the mouse (160-163), while only a minority of individuals show imprinting of the *IGF2*-receptor with exclusive expression of the maternal allele (164-166). *H19* and *IGF2* are localized on the short arm of chromosome 11, band 15.5, approximately 200 kb apart (167). Both genes are important in early development (156,168,169). Murine complete androgenotes show expression of both *H19* and *igf2*, while gynogenotes lack expression of both genes (170). A tumor suppressor activity for the *H19* gene was recently suggested (171). Disturbance of the monoallelic pattern of expression, referred to as relaxation of imprinting or loss of imprinting (LOI), has been reported in human renal Wilms' tumors and in one rhabdoid tumor (172,173). Biallelic expression of the *IGF2* gene is found in the majority of cases, and in a minority for *H19*. In addition, LOI has been reported in approximately 40% of lung cancers (174). In contrast, retention of uniparental expression of *IGF2* is reported in hepatoblastoma (175). No studies have been done on allele specific expression of the *IGF2* and *H19* genes in TGCTs.

The mechanism(s) involved in the specific expression of either the maternal or paternal allele of imprinted genes is not yet elucidated. That DNA methylation plays a role is illustrated by mice deficient in DNA methyltransferase activity (176). These mutants die in utero around day 11 postcoitum. They show expression of both parental alleles of *H19*, and no expression of the *igf2* and *igf2*-receptor genes (177,178). This is in agreement with the different methylation pattern of these genes during normal development in relation to their expression (179-182). *H19* is expressed from the demethylated allele, while the opposite is found for *igf2* and its receptor. These data illustrate that methylation is required for monoallelic expression of the imprinted genes, and that it may have opposite effects.

Changes in the methylation status of murine and human primordial germ cells during their maturation to mature gametes follow different patterns in the paternal and maternal germ line (183-188). Female murine germ cells remain demethylated, while male germ cells become methylated postnatally during spermatogenesis. It is unknown thus far whether the primordial germ cells escape de novo methylation just before gastrulation, or are demethylated and remethylated in a sex dependent pattern. Obviously primordial germ cells are overall hypomethylated, but it is unknown whether this is associated with loss of the non-equivalence and/or differential expression of imprinted genes.

Based on the study of autosomal and X-chromosomal genes a hypomethylated status for SE compared to NS was reported (189). Whether this is due to evolution of the

cancer (190), or an inherent characteristic of that cancer c.q. of its cell of origin, is not known. The parent dependent methylation polymorphism on chromosomal region 15q11-q13 might offer a tool to study this problem (191-193). The approach is based on the detection of differences between the methylation status of the paternally and maternally derived chromosomal regions. Using this technique, the bi- or uniparental methylation pattern of TGCTs can be studied, which may be indicative for the GI status of the cell of origin of this cancer.

Paragraph 6.

PATHOGENESIS OF GERM CELL TUMORS OF THE ADULT TESTIS.

The incidence of TGCTs shows a dip during the early sixties in men conceived and born during and immediately after World War II, most probably due to a suppressing effect of World War II (194). This indirectly suggests an intrauterine initiation of the development of TGCTs. The hypothesis is that the unborn boys were as a result of the sober life style during the war protected against oestrogen (over)exposure in utero. This would be in agreement with the hypothesis of Henderson (195), suggesting that the higher testosteron level in black women during pregnancy pushes the differentiation of early germ cells to mature gonocytes. Besides the lower overall incidence, the epidemiological characteristics of TGCTs in blacks are similar to those in whites, except the SE are clinically manifest at an earlier age (196). A lack or delay of maturation of (primordial) germ cells to mature gonocytes may be an important mechanism in the pathogenesis of TGCTs, as recently suggested for most cancers (197). This assumption of a very early initiation of TGCTs is supported by the finding of alkaline phosphatase, 43-9F and tra-1-60 expression in the testis of boys under one year of age, without any indication of malignancy (198), and by the similarities between CIS cells and primordial germ cell (I.3 and I.5). The cell of origin of TGCTs might even be initiated at a stage before it migrates to the gonadal blastema. This hypothesis could elegantly explain the relatively high figure (5-10%) of bilaterality in TGCTs (199, for review).

The events involved in initiation, promotion and progression of TGCTs are still largely unidentified (200). Cryptorchidism is an established, important risk factor (19, for review) and the activity of the pituitary-testis axis during puberty may be involved (201), as suggested by the finding of androgen receptors on SE and CIS (202). Atrophy of the testis, possibly induced by viral infections, is also suggested as a promoting factor (203).

The consistent aneuploidy of invasive TGCTs as well as CIS (I.4.2 and I.4.3) strongly suggests that polyploidization is an early step in the pathogenesis of this cancer, of which the trigger and the mechanism (endoreduplication or cell fusion) are still unknown.

Chromosomal analyses have prompted new ideas about the development of TGCTs. No higher constitutional genomic instability compared to a control population is found for patients with a TGCT (204), and no chromosomal germ line aberrations are found in patients with a bilateral or familial TGCT (205). Because of the presence of both the X and Y chromosome in TGCTs, they are assumed to be of premeiotic origin (206, I.4.3).

The consistent overrepresentation of the short arm of chromosome 12, mainly as i(12p) (I.4.3), suggests that 12p overrepresentation is a crucial event. The finding that heterozygosity of the long arm of chromosome 12 is retained (122,123,152,153) indicates that polyploidization precedes i(12p) formation. For the formation of i(12p) two mechanisms have been proposed: nonreciprocal interchange between centromeres of nonsister chromatids (207), and exchange between sister-chromatids (152,153). Recently a model for the development of TGCTs has been proposed, assuming initial loss of activity of a tumor suppressor gene or genes on the long arm of chromosome 12, and subsequent N-*RAS* mutations (136,208).

It is well established, both by in vivo and in vitro studies, that embryonal and extraembryonal tumor components are derivatives of totipotent EC cells (197,209, for review). Histologically this is supported by the presence of complete or incomplete embryoid bodies in more than 50% of the mixed NS (210). Very little is known about the relationship between CIS, SE and NS. Besides some epidemiological and immunohistochemical data, no evidence for the presence of one common, or two (or more) independent precursors for SE and NS is available. Therefore, generally speaking two different pathogenetic models are possible, schematically illustrated in Figure 5.

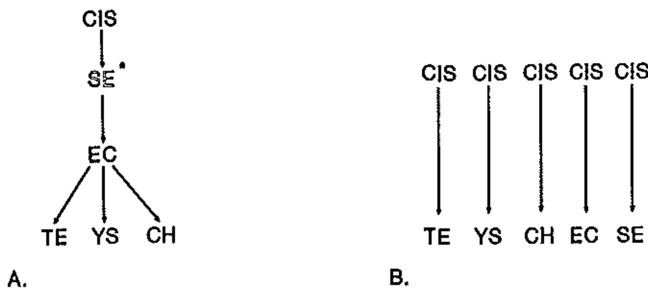

Fig. 5. Schematic representations of the different pathogenetic models for testicular germ cell tumors of adults; (A) linear progression model; (B) independent origin model.

Model A, originally proposed by Ewing in 1911 who stated "its (SE) teratomatous relations are, therefore, not a matter of assumption, but a readily demonstrable fact" (211), and further developed by others (10,37,212,213), suggests that SE and NS have a common CIS as precursor, and that SE is an intermediate stage between CIS and NS. The similarities in over- and underrepresentation of chromosomes between the different invasive TGCT subtypes, the consistent presence of chromosome 12 abnormalities, and the common chromosomal aberrations in SE and NS components of CTs support this hypothesis. In addition, immunohistochemical studies of SE, showing NS differentiation (so called intermediate phenotypes), isolation of NS cell lines with SE characteristics, HLA studies and the occurrence of NS metastases after treatment of a pure SE (37,214-225) are also suggestive for this model. In contrast to this so called "linear progression model", a second theory (model B) favors independent populations of CIS for SE and NS

(226-229). These different CIS populations can theoretically be derived from one common precursor. In the latter model the transition of SE into a NS is not possible. In the "linear progression model", on the other hand, NS always develops from a SE-precursor, either CIS or, less often invasive SE.

Because of the inconsistency in the chromosomal and molecular data obtained by the different research groups, no convincing evidence for one of the models is available yet.

Paragraph 7.

OUTLINE OF THE THESIS.

The above review on TGCTs illustrates that multiple targets and mechanisms are assumed in the development of this cancer, including imbalances between different (parts of) chromosomes, loss of tumor suppressor gene activity, and activation of proto-oncogenes. Besides the aneuploid DNA content and the specific overrepresentation of the short arm of chromosome 12, no consistent aberrations pinpointing important events in the pathogenesis of TGCTs have been obtained in recent years, in spite of considerable efforts. In this thesis, three main goals can be distinguished.

1. Study of the relationship between the precursor CIS and the different types of invasive TGCTs:

Epidemiological, clinical, morphological, immunohistochemical, ploidy, and chromosomal data suggest a close relationship between different NS components, between NS and SE components within a CT, and between CIS and the adjacent TGCT. Invasive TGCTs show a more or less consistent chromosomal constitution, with some intriguing characteristics, i.e., the specific involvement of chromosome 12 and the overrepresentation of chromosome 15 in SE compared to NS. It is not known whether this difference is due to karyotyping of selected subclones of NS. Besides the hypertriploidy of CIS, little is known about the chromosomal constitution of CIS, and how it compares to the adjacent invasive TGCT.

The study of the relationship between CIS, SE and NS on the basis of phenotypic characteristics is troublesome because the cancer cells may show a phenotypical spectrum due to their developmental potential, not correlating with progression. Therefore genomic analyses have been performed, including DNA ploidy and karyotypic studies. In our hands SE and CIS can be karyotyped only by direct harvesting of metaphases and NS virtually only after short term culture in vitro. Both methods carry the risk of selection for subpopulations of malignant cells, moreover the success rate for karyotyping of CIS is very low thus far.

For our comparative studies interphase cytogenetics on tissue sections is ideally suited, in particular when combined with immunohistochemistry to label the cells of interest. At the beginning of these studies, no suitable probe for ISH specific for the centromeric region of chromosome 12 was available. Therefore one was isolated (Chapter II and 230). In addition, we have used commercially available probes for the centromeric regions of the chromosome 1 and 15 (Chapters III, IV and IX).

2. Study of molecular aberrations in TGCTs:

On the basis of data from the literature suggesting involvement of the short arm of chromosome 11 and the *WT1* gene in TGCTs, the involvement of deletions affecting 11p13 and/or 11p15.5 and aberations within exons 2 and 6 and the zinc finger regions of *WT1* were studied (Chapter V). In addition, analysis of deletions of two loci on the long arm of chromosome 16 was included, because of their putative role in some renal Wilms' tumors (231). In this context a Wilms' tumor of the testis was studied (Chapter IX).

The chromosomes 12, 15 and 22 were also studied (Chapters VI and VIII). Chromosome 12 because of the conflicting data on the role of deletions affecting two loci on the long arm, and chromosome 15 and 22 in view of the karyotypically found overrepresentation in SE compared to NS.

3. Study of phenomena indicative for a role of GI in TGCTs:

Evidence for GI was looked for by analyzing the parental origin of the affected regions of the chromosomes showing allelic imbalances. Allelic expression of the two imprinted human genes *H19* and *IGF2* was studied (Chapter VII). In addition, the methylation status of chromosomal region 15q11-q13, identified by the probe DN34, showing parental origin differences, was investigated.

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Chapter II.

Localization and Polymorphism of a Chromosome 12-Specific α Satellite DNA Sequence.

Cytogenetics and Cell Genetics 53: 216-218, 1990.

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ABSTRACT

The isolation and localization of a chromosome 12-specific *α* satellite DNA sequence, p*α*12H8, is described. This clone contains a complete copy of the 1.4-kb *Hin*dIII higher-order repeat present within the *α* satellite array on chromosome 12. The specificity of p*α*12H8 was demonstrated by *in situ* hybridization and Southern blot analysis of a somatic cell hybrid mapping panel, both performed under high-stringency conditions. Polymorphic restriction patterns within the *α* satellite array, revealed by the use of the restriction enzymes *Bgl*II and *Eco*RV, were demonstrated to display Mendelian inheritance. These properties make p*α*12H8 a valuable genetic marker for the centromeric region of chromosome 12.

INTRODUCTION

Human *α* satellite DNA (alphoid DNA) is a well defined heterogeneous family of repetitive DNA sequences. The fundamental repeat unit is approximately 170 bp in length and is found in long tandem arrangements within the centromeric region of all human chromosomes (Mitchell et al., 1985). On a number of different chromosomes, *α* satellite DNA can be characterized by highly specific long-range restriction site periodicities which define distinct multimeric higher order repeat units superimposed on the 170-monomer array (Willard and Waye, 1987, and references cited therein).

When this characteristic higher-order repeat is used as a probe under the proper stringency conditions in either a Southern or an in situ hybridization study, it will detect only its cognate chromosome (Cremer et al., 1986; Willard et al., 1986; Devilee et al., 1988a). At present, chromosome-specific *α* satellite repeats are available for about 14 different chromosomes, including the X and Y chromosome. These probes have proven useful for the diagnosis of chromosomal rearrangements by in situ hybridization of metaphase spreads, as well as for the detection of numerical aberrations in interphase nuclei (Cremer et al., 1986; Devilee et al., 1988b). Further, some of these probes detect polymorphic restriction patterns within the *α* satellite array and may thus serve as valuable genetic markers of their centromeric regions (Willard et al., 1986).

Thus far, a chromosome 12-specific *α* satellite DNA probe has not been available. Our interest in such a probe stems from its potential application in our ongoing study of the involvement of chromosome 12 in the pathogenesis of germ cell tumors (Castedo et al., 1989).

MATERIALS AND METHODS

We set out to characterize a human/hamster somatic cell hybrid A3/TE-7B (which was kindly given to us by Dr. A. Geurts van Kessel, University of Nijmegen) for the presence of an *α* satellite higher-order repeat. Cytogenetic analysis demonstrated that the centromere of chromosome 12 is the only human centromere present in this cell line (data not shown). Genomic DNA from A3/TE-7B was digested with 20 different restriction enzymes, size-fractionated, blotted onto nylon filters, and hybridized with p*α*3.5, an *α* satellite probe from chromosome 3 (Waye and Willard, 1989), under conditions allowing considerable cross-hybridization (Willard, 1985). A 1.4-kb band was generated by four different enzymes, though most pronounced by *Hin*dIII and *Pvu*II (data not shown). A few additional less intense bands, corresponding in length to multiples of 340 bp, were apparent in these digests. *Hae*III and *Hin*fI both yielded strong bands at the 340-bp position, whereas a single band of 680 bp was seen in an *Eco*RI-digest (data not shown). This indicates that the *α* satellite array on chromosome 12 basically has a dimeric organization, with longer-range restriction site periodicities detected by both *Hin*dIII and

*Pvu*II. This higher-order repeat of 1.4-kb can be predicted to consist of eight tandem 170-bp alphoid monomers (8-mer). A similar organization of alphoid DNA on chromosome 12 was recently also reported by others (Baldini et al., 1990).

A λ phage (Lambda ZAP II cloning kit, Stratagene) library was constructed from the 1.3- to 1.5-kb fraction of *Hin*dIII-digested DNA of A3/TE-7B. The library, consisting of approximately 30,000 phages, was screened with pα3.5 under low stringency conditions. Two positive phages were identified, and one was excised into the pBluescript vector according to the manufacturer's directions. The resulting recombinant plasmid (pα12H8) was used throughout this study.

RESULTS AND DISCUSSION

Localization of the 1.4-kb *Hin*dIII insert of pα12H8 was determined by high-stringency filter hybridization of *Hin*dIII-digested DNAs isolated from a panel of human x rodent somatic cell hybrids (Fig. 1).

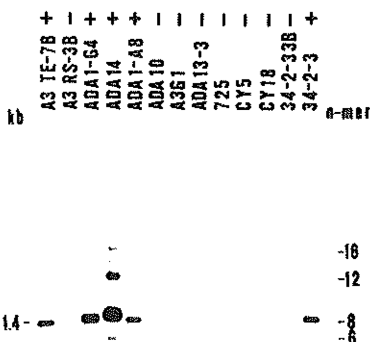

Fig. 1. Localization of the 1.4-kb HindIII insert of pα12H8 to chromosome 12 by somatic cell hybrid mapping. Genomic DNA from the cell hybrids was digested with HindIII, size fractionated on a 0.7% agarose gel, tranferred to nylon filters, and hybridized with pα12H8 under high-stringency conditions (Devilee et al., 1988a). Overnight was exposure. Presence or absence of chromosome 12, as determined by cytogenetic analysis, is indicated by (+) or (-) above each lane.

Only those hybrids that contained chromosome 12 showed a strong signal at the 8-mer position (1.4-kb). Hybrids with very strong 8-mer signals showed additional less intense bands, with lengths corresponding to multiples of 340 bp. Thus, in a total of 20 hybrids analyzed, 90% of the cell lines were consistent with the assignment of the pα12H8-homologous repeats to chromosome 12. In situ hybridization of pα12H8 under high-stringency conditions to metaphases of lymphocytes from a healthy donor resulted in strong, specific labeling of the centromeric regions of two chromosomes (Fig. 2 on color plate). Giemsa-banding performed prior to in situ hybridization (Smit et al., 1990) identified these as both homologs of chromosome 12 (Fig. 3). Note that the interphase nucleus shown in Fig. 2 contains two distinct spots, allegedly reflecting the presence of both chromosomes 12. This was found to hold true for more than 90% of the approximately 200 nuclei examined (data not shown).

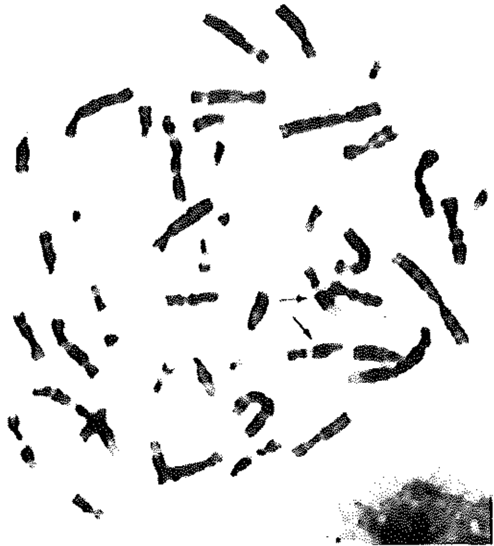

Fig. 3. Giemsa-banding of the same metaphase spread shown in Fig.2. Arrows point to two chromosomes 12 located in the same positions as the brightly fluorescing spots in Fig. 2.

Given the extreme size of the array of alphoid DNA on chromosome 12 (approximately 10^6 bp, our unpublished copy-number estimation), the distribution of restriction sites of endonucleases that cut infrequently within the array may be expected to be polymorphic in the population (Willard et al., 1986). Such restriction enzymes are predicted to produce a prominent unresolved band of more than 40 kb, representing the bulk of uncut *α* satellite DNA, plus a series of less intense polymorphic fragments in the order of 2-20 kb (*α* satellite "morphs"; see Waye et al., 1987, for nomenclature). The clone p*α*12H8 detects such patterns when hybridized under high-stringency conditions to total human genomic DNA digested with either *Bgl*II or *Eco*RV. These patterns were found to be polymorphic in a group of nine unrelated individuals (Fig. 4).

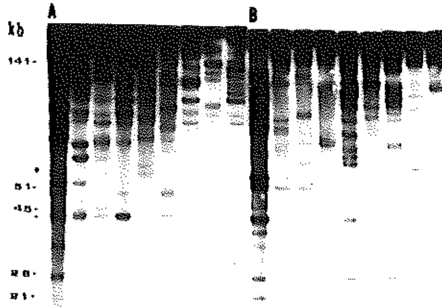

Fig. 4. Detection of α satellite "morphs" on chromosome 12. Genomic DNA from nine unrelated individuals was digested with BglII (a) or EcoRV (b); experimental procedures as described in the legend to Fig. 1.

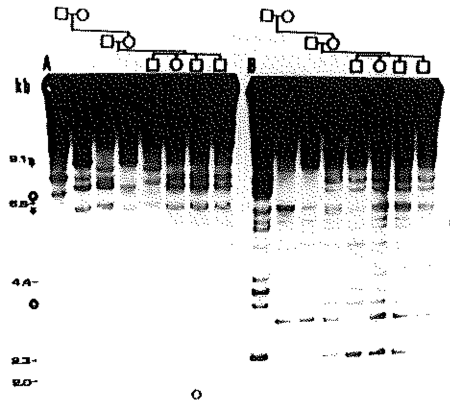

Fig. 5 Mendelian inheritance of α satellite "morphs" on chromosome 12. Relationship between siblings is indicated. Experimental procedures as described in the legend to Fig. 1, using BglII (a) and EcoRV (b) as restriction enzymes.

Some fragment lengths appear relatively frequently, while others are relatively rare. A 4.3-kb *Bgl*II- and a 3.2-kb *Eco*RV fragment are present in 9/9 individuals (arrows in Fig. 4), whereas a 7.0-kb *Bgl*II and a 13-kb *Eco*RV fragment are present in 1/9 individuals (stars in Fig. 4). Studies of genotype frequencies of similar polymorphisms of the α satellite arrays on chromosome 10 and the X chromosome have indicated that even the most frequent α satellite "morphs" in the population derive from a single chromosome in at least 80 % of the individuals who are positive for it (Willard et al., 1986; Devilee et al., 1988a). Mendelian inheritance of chromosome 12 α satellite "morphs" was established in three two- and two three-generation pedigrees. Segregation of both *Bgl*II and *Eco*RV "morphs" within a three-generation pedigree is shown in Fig.5. A number of bands (stars in Fig. 5) can be seen to cosegregate in the family and thus to form a haplotype.

In this report, we have described the isolation and mapping of a chromosome 12-specific alphoid repeat, designated pα12H8. We demonstrated the potential of this probe in detecting numerical aberrations by in situ hydridization of interphase nuclei, as well as in identifying chromosomal rearrangements in metaphase spreads. The described polymorphism of the α satellite array may be useful in developing a centromere-based genetic linkage map of chromosome 12. We are presently applying pα12H8 in the investigation of the role of chromosome 12 and its aberrations in the pathogenesis of germ cell tumors.

Acknowledgments

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Chapter III.

In situ Numeric Analysis of Centromeric Regions of Chromosomes 1, 12, and 15 of Seminomas, Nonseminomatous Germ Cell Tumors, and Carcinoma *In situ* of Human Testis.

Laboratory Investigation 68: 211-219, 1993.

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ABSTRACT

BACKGROUND: No detailed data are available concerning the possible chromosomal heterogeneity within testicular germ cell tumors of adults (TGCTs). In addition, little is known about the chromosomal constitution of carcinoma *in situ* (CIS) of the human testis, the precursor of TGCTs, and the possible relation with the different histological tumortypes (seminomas (SE), nonseminomatous TGCTs (NS)).

EXPERIMENTAL DESIGN: Interphase cytogenetics in combination with immunohistochemistry was performed on tissue sections of SE, NS and their adjacent CIS to study the numerical distributions of centromeric regions of chromosomes 1, 12, and 15.

RESULTS: No differences in chromosomal constitution were found between CIS adjacent to SE and the invasive tumor itself. NS showed a significant lower number of copies of chromosome 15 than SE, which was also found for the adjacent CIS. In addition a significantly higher number of copies of chromosome 12 was found in CIS adjacent to SE compared with CIS adjacent to NS. Invasive NS showed a significantly higher chromosome 1 and 12 copy number compared with its adjacent CIS.

CONCLUSIONS: Net loss of chromosomes during tumor evolution can explain the differences between SE and NS and also between their adjacent CIS. Based on these results, we hypotheze that CIS-SE may progress not only into SE, but also into CIS-NS. It is conceivable, therefore, that NS may evolve from SE or from CIS-NS.

INTRODUCTION

Carcinoma *In Situ* (CIS) of the human testis is generally accepted as the precursor of all testicular germ cell tumors of adults (TGCTs) except spermatocytic seminoma (1). The lesion is frequently identified adjacent to invasive TGCTs (2, 3), has a characteristic morphology (reviewed in Ref. 4) and is aneuploid (5-7 and references cited therein). Besides limited karyotypic (8) and interphase cytogenetic data (9, 10), little is known about its chromosomal constitution. Questions concerning the early chromosomal and molecular events in the pathogenesis of TGCTs, possible chromosomal heterogeneity, and differences between CIS adjacent to the different histological tumor types [seminomas (SE), nonseminomatous TGCTs (NS), and combined tumors] remain to be answered. Heterogeneity within the invasive tumor itself is in some cases apparent from multiple stem-lines detected by DNA flow cytometry and/or image analysis (IA) (11-15), but the differences in chromosomal constitution of these tumor cell subpopulations are unknown.

To study these problems in more detail, we applied immunohistochemistry in combination with non-radioactive *in situ* hybridization (ISH) using centromere specific repetitive DNA probes on 20-μm frozen tissue sections. Placental-like alkaline phosphatase (PLAP) expression was used to identify CIS and SE cells (16, 17), and cytokeratin expression for the identification of NS cells (18, 19 and references cited therein). Because of the frequent involvement of chromosomes 1 and 12 in numerical and structural abnormalities in TGCTs, as well as the cytogenetic data revealing a significantly higher copy number of chromosome 15 in SE compared with NS (reviewed in Ref. 20), we investigated the numerical distributions of centromeric regions of these chromosomes in six SE and five NS, as well as their adjacent CIS (CIS-SE and CIS-NS, respectively). Ploidy of these different components was determined by DNA flow cytometry.

EXPERIMENTAL DESIGN:

ISH using chromosome specific centromeric repetitive DNA probes in combination with immunohistochemistry on tissue sections allows analysis of the chromosomal

composition of specific cell populations in their histological context. Using this approach, we studied the numerical distribution pattern of chromosomes 1, 12 and 15 of six SE and five NS, as well as CIS within the same tumor-bearing testis. To identify CIS and SE, PLAP expression was used as immunohistochemical marker, whereas leucocyte common antigen (LCA) was also used as nontumor specific marker in SE. Cytokeratin 8 and 18, specifically recognized by the monoclonal antibody CAM 5.2, was used as NS specific marker. The numerical distribution of centromeric regions of chromosome 1 [identified by pUC 1.77, (21)], chromosome 12 [identified by pα12H8, (22)], as well as chromosome 15 [identified by D15Z1, (23)] was studied using this approach. The possibility of biased results caused by analysis of partial nuclei and/or restricted probe penetration, due to the thickness of the tissue section, was analyzed by comparison of results obtained by using 1) an imprint preparation, 2) a 6-μm frozen tissue section, and 3). a 20 μm frozen tissue section, respectively, all derived from one SE. These were processed as described, using pUC 1.77 as probe and PLAP as positive identification marker. Screening was performed by two persons, who scored only nuclei which appeared visually intact. The data obtained by the two investigators did not differ significantly (< 5%), and are summarized in Figure 1.

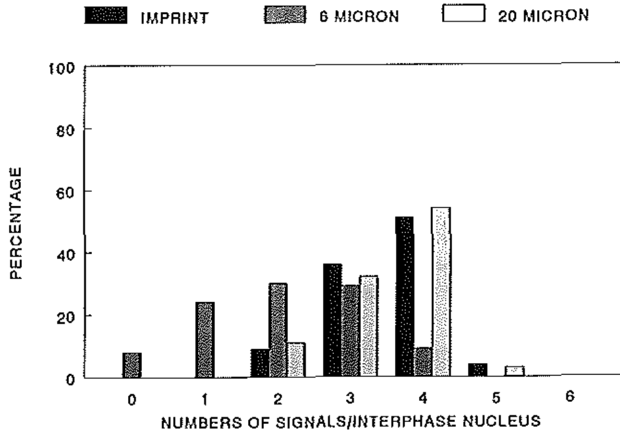

Fig. 1. Schematic of influence of using single cell suspensions or 6-μm and 20-μm tissue sections on distribution of centromeric regions of chromosome 1 in placental like alkaline phosphatase positive SE (n = 100).

These results indicate that 20-μm tissue sections allowed reliable selection of whole nuclei and showed satisfactory penetration of the probe [using a 6-μm tissue section results in a significant shift to the left compared with using an imprint preparation or a 20-μm tissue section ($p < 0.001$, chi-square test)].

To determine if the numbers of ISH signals do correlate with the centromeric copies present in aneuploid nuclei, this protocol was used on a cytogenetically characterized SE. Based on the karyotype (Table 1) three signals of chromosome 1 and five signals of chromosome 12 centromeric regions were expected. The ISH results (in percentages) using pUC 1.77 and pα12H8 are summarized in Table 1.

The PLAP positive cells showed for chromosome 1 three signals/interphase nucleus in 74% and for chromosome 12 five signals/interphase nucleus in 92%. Similar data were obtained using LCA as identification marker and counting the numbers of signals in the LCA negative cells (not shown). Because a tumor cell specific marker facilitates the discrimination between individual tumor cells, PLAP was used as identification marker for SE and CIS cells throughout this study.

Table 1. ISH RESULTS

Chromosome	Cytogenetics	Number Signals/Interphase Nucleus 0	1	2	3 %	4	5	No. of cells
1	#1,#1,der(1) t(1;?)(1q14;?)	0	0	26	74	0	0	1011
12	#12,#12,i(12p) i(12p),der(12) t(12;?)(12p13;?)	0	0	0	0	8	92	826

Cytogenetics of chromosomes 1 and 12 and data of ISH using pUC 1.77 and pα12H8 on 20-μm tissue sections of a seminoma, with placental like alkaline phosphatase expression as selection criteria.

Counting of the CIS and tumor specimens was performed by one person, while at random results were checked by another. Proper hybridization was verified by counting the number of signals in nontumor cells (cells of the spermatogenic lineage, stromal-, Sertoli- and/or Leydig cells; i.e., PLAP negative cells in CIS containing testis tissue and SE). In NS cytokeratin negative cells were used as internal quality control. Analysis of the tumor cells was only performed if at least 95% of 300 nuclei of the nontumor cells contained the number of expected signals: two for diploid and one for haploid cells. If not, the procedure was repeated.

Heterogeneity of signal distributions per nucleus between different seminiferous tubules within one specimen and different locations within the invasive tumor (when available) was analyzed separately for each specimen. For each specimen and centromeric region a $n \times (m+1)$ table was created where n is number of locations and $m=5$ the maximum number of spots. The value of a cell of this table (e.g., row i, column j) was defined as the number of cells with j spots at location i. The chi-square test of independence was applied to this table to detect heterogeneity between the rows (locations). Signal distributions per specimen were summarized by the mean number of spots/nucleus (MNSN) and the associated standard deviation. Differences between the MNSN of CIS-SE and CIS-NS, SE and NS were analyzed using the paired and unpaired Student's *t* test. DNA flow cytometry was performed as described to identify the ploidy of the CIS and tumor cells (14, 24).

RESULTS AND DISCUSSION

In contrast to the rapidly growing list of chromosomal and molecular data on invasive TGCTs (reviewed in Ref. 20, 25-30) little is known about CIS, considered to be their precursor. Karyotyping of these cells is troublesome (8), because tissue culture has not been successful thus far, and direct harvesting is difficult because of the small number of available cells, which are enclosed in the seminiferous tubule and thus hard to dislodge.

It is not known whether the chromosomal constitution of CIS is homogeneous or heterogeneous within one testis or if the karyotype differs depending on the presence and/or histological type of invasive tumor. Chromosomal heterogeneity of the invasive tumor components is also not studied in detail thus far, but ploidy data suggest that it is present is some cases (11-15, 31).

Morphologically and immunohistochemically (4 and references cited therein, personal observation) CIS resembles SE, whereas NS seems to be composed of rather homogeneous cell populations. Image analysis of CIS from eight infertile men without a

TGCT displayed a peritetraploid mean ploidy value (32). Using the same approach, maldescended testes contained in 17/19 cases germ cells with an abnormal DNA content, without a clear clustering around the tetraploid range (33). Another study described the presence of CIS in five out of 723 infertile men showing all a peritetraploid DNA content (7). Aneuploidy of CIS is described (6, 31, 34). Using flow cytometry the DNA content of CIS-SE and CIS-NS adjacent to the invasive tumor has been studied. It was not different in the two types of CIS with the same DNA content as SE (5). Two independent studies illustrated the aneuploidy of centromeric regions of chromosome 1 in CIS (9, 10).

To study the chromosomal constitution of CIS and invasive tumor cells in more detail, we used nonradioactive ISH, referred to as "interphase cytogenetics" (35), in combination with immunohistochemistry for the detection of subpopulations of cells on tissue sections. The reliability of this approach was tested using whole cell suspensions and tissue sections of different thickness (Fig. 1), as well as on 20-μm thickness slides of a cytogenetically characterized SE (Table 1). These data illustrate that frozen 20-μm tissue sections in combination with PLAP detection revealed proper hybridization and allowed analysis of whole nuclei.

This approach was used for screening the numerical distribution patterns of chromosomes 1, 12 and 15 centromeric regions in CIS (n = 11) and SE (n = 5). Cytokeratin was used as marker of tumor cells in NS (n = 6). Representative results are illustrated in Figure 2 (color plate), using pα12H8, identifying the centromeric regions of chromosome 12 in normal cells of the spermatogenic lineage (note the haploid ones) (A), and in PLAP expressing CIS (B).

No localization dependent distribution pattern were found between different CIS containing seminiferous tubules within one specimen in 28 of the 33 cases (not shown). When different localizations within a tumor were investigated also no differences in distributions were found (not shown). Therefore, the MNSN within one specimen is taken as representative for that particular specimen. These data are summarized in Table 2 and schematically illustrated in Figure 3. Statistical analysis is summarized in Table 3.

Table 3. Statistics

	Paired Student's t test		*Unpaired Student's t test*	
Chromosome	NS/CIS-NS	SE/CIS-SE	CIS-NS/CIS-SE	NS/SE
1	0.03	ns	ns	ns
12	0.006	ns	0.014	ns
15	ns	ns	0.014	0.003

Statistical analysis of the in situ hybridization data, showing p values if significant differences were detected. ns, not significant (p > 0.17).

The cytogenetic finding of lower copy numbers of chromosome 15 in NS compared with SE (reviewed in Ref. 20) is supported by our ISH results, showing a lower MNSN for NS than for SE ($p < 0.003$). No differences concerning chromosomes 1 and 12 were found between SE and NS. CIS-SE and SE showed a similar chromosomes 1, 12 and 15 distribution pattern, whereas NS displayed a significant higher MNSN of chromosomes 1 and 12 than CIS-NS ($p < 0.03$ and $p < 0.006$). CIS-NS showed a significantly lower MNSN of chromosomes 12 and 15 compared to CIS-SE ($p < 0.014$).

The fact that these differences are not detected by DNA flow cytometry (Table 4) may have several explanations, e.g., tetraploid CIS cells (4 signals for every autosome) are not revealed by DNA flow cytometry, because they are obscured in the G2/m peak;

DNA flow cytometry is not sensitive enough to detect these relatively small chromosomal differences; gain or loss of (centromeric regions of) chromosome 1, 12, and 15 may be compensated by the loss or gain of other chromosomes or chromosomal regions. This latter possibility is supported by the idea that gain and loss of chromosomes during tumor progression is random (36).

Table 4. DNA index of SE and NS and their adjacent CIS-SE and CIS-NS

Specimen	DI	Specimen	DI	Specimen	DI	Specimen	DI
CIS-S1	1.62	SE1	1.62	CIS-NS1	1.55	NS1	1.43
CIS-S2	1.73	SE2	1.50	CIS-NS2	1.60	NS2	1.50
CIS-S3	1.62	SE3	1.55	CIS-NS3	1.57	NS3	1.46
CIS-S4	1.51	SE4	1.46	CIS-NS4	1.51	NS4	1.57
CIS-S5	1.55	SE5	1.55	CIS-NS5	1.63	NS5	1.41
CIS-S6	1.63	SE6	1.60				

Specimens analyzed by single parameter DNA flow cytometry using separately processed components. DI, DNA index.

Recent data on interphase cytogenetics using 6-μm paraffin embedded tissue sections show one (24%), two (41%), and three (34%) chromosome 1 centromeric region signals per CIS nucleus (372 nuclei of five different cases) (10). In our series of 11 cases only 8% of 10057 CIS nuclei showed one signal. This illustrates the advantages of using ISH in combination with immunohistochemistry on 20-μm tissue slides to analyze the chromosomal constitution of certain cell populations within their histological context. ISH with chromosome specific probes (in particular for chromosome 1) has been suggested as a valuable tool for the diagnosis of CIS in seminal fluid (9). However, our data show that CIS with two signals/nucleus for centromeric regions of chromosome 1 can be frequently found [in the range of 0% (case CIS-NS 3) to 62% (case CIS-SE 5) comparable with the data of the invasive TGCTs (0% for NS 3 and 61% for SE 5) (Figure 3)]. This implies that numerical analysis of chromosome 1 centromeric regions only will lead to an underestimation of the prevalence of CIS.

Our results of combined immunohistochemistry and ISH show that CIS-SE differs from CIS-NS with respect to its chromosomal constitution, while they are similar in morphology, immunohistochemistry, and ploidy. The mechanism of net loss of chromosomes (after polyploidization), suggested to be part of the karyotypic evolution of invasive TGCTs (20, 14), can explain the differences found between CIS-SE and CIS-NS. CIS-SE can progress into an invasive SE without gross chromosomal changes. Alternatively progression of CIS-SE to CIS-NS may occur (associated by loss of copies of chromosomes 12 and 15). NS can evolve from SE (associated by loss of copies of chromosome 15) or from CIS-NS (associated by a gain in chromosomes 1 and especially 12). This implies that CIS may go through a similar kind of evolution as SE. The question if SE and NS arise from independent CIS or according to a linear progression model with a common CIS (see Ref. 37 for discussion) is still unanswered.

Additional studies will be performed using this approach to study the distributions of chromosomes 1, 12, and 15 on more CIS specimens (including CIS adjacent to combined tumors), as well as their adjacent invasive tumors. Also, distribution of other chromosomes will be investigated, especially chromosome 22, which like chromosome 15 is underrepresented in NS compared with SE (20). Based on the data presented here, the hypothesis that chromosomal and/or molecular aberrations detected in CIS localized adjacent to an invasive TGCT are by definition early events in the

pathogenesis of TGCTs must be reconsidered. Therefore CIS detected in the preinvasive tumor stage will be studied to pinpoint the early chromosomal events involved in the pathogenesis of these TGCTs. At variance with results of Mukherjee *et al.* (30), using D12Z3 as probe instead of pα12H8, we found no differences in shape or size of the signals of the centromeres in i(12p) and normal chromosomes 12. Therefore, the absence or presence of i(12p) in CIS as well as in rare germ cell tumors will be studied using the technique presented in this paper, in combination with double hybridization with chromosome 12p-specific cosmids and YACs.

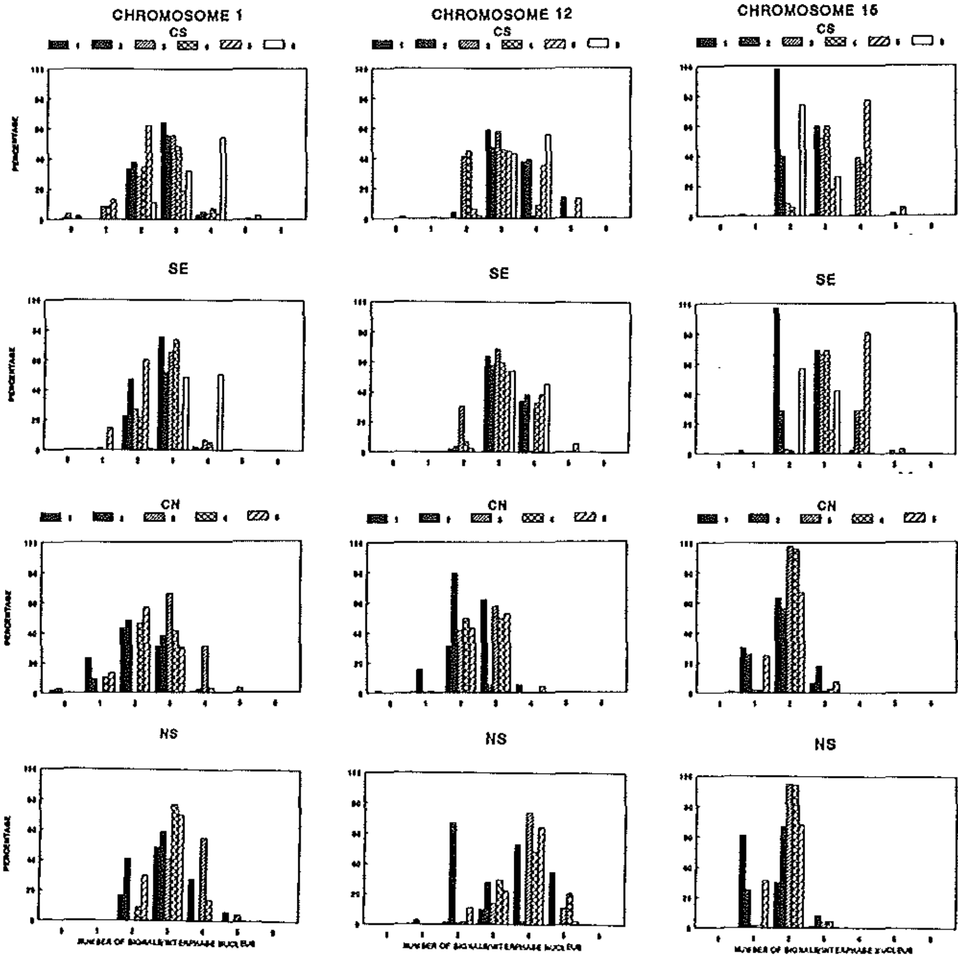

Fig. 3. Schematic of distributions (percentages) of centromeric regions of chromosomes 1, 12, and 15 in the nuclei of tumor cells of six seminomas (SE), five nonseminomatous testicular germ cell tumors (NS), as well as their adjacent carcinoma in situ (CS) and (CN). Tumor cells are defined immunohistochemically by placental like alkaline phosphatase (SE and CIS) or cytokeratin expression (NS).

Table 2. Data summary

CASE	$n^{loc.}$	n^{cells}	MNSN	CASE	$n^{loc.}$	n^{cells}	MNSN	CASE	n^{loc}	n^{cells}	MNSN	CASE	n^{loc}	n^{cells}	MNSN
Chromosome 1															
CIS-SE1	17	1442	2.7±0.26	SE1	1	633	2.8±0.20	CIS-NS1	22	1253	2.0±0.63	NS1	1	327	3.3±0.65
CIS-SE2	13	782	2.6±0.44	SE2	3	955	2.5±0.27	CIS-NS2	13	805	2.3±0.61	NS2	1	78	2.6±0.24
CIS-SE3	15	545	2.5±0.72	SE3	1	1331	2.8±0.33	CIS-NS3	7	198	3.4±0.30	NS3	1	2523	3.6±0.32
CIS-SE4	15	1338	2.6±0.62	SE4	1	1231	2.8±0.24	CIS-NS4	13	823	2.4±0.48	NS4	1	1834	3.1±0.23
CIS-SE5	14	639	2.1±0.55	SE5	1	1444	2.1±0.38	CIS-NS5	17	1275	2.2±0.40	NS5	1	172	2.7±0.21
CIS-SE6	11	957	3.5±0.52	SE6	1	1290	3.5±0.27								
Total	85	5703	2.7±0.46		8	6884	2.7±0.41		72	4354	2.4±0.53		5	4934	3.0±0.42
Chromosome 12															
CIS-SE1	11	1294	3.3±0.30	SE1	1	982	3.3±0.26	CIS-NS1	14	1107	2.7±0.41	NS1	1	487	4.2±0.48
CIS-SE2	9	396	3.7±0.50	SE2	2	338	3.3±0.30	CIS-NS2	10	441	1.9±0.20	NS2	1	361	2.3±0.30
CIS-SE3	12	939	2.6±0.27	SE3	1	1354	2.7±0.22	CIS-NS3	6	465	2.6±0.26	NS3	3	702	4.0±0.28
CIS-SE4	14	1133	2.6±0.45	SE4	1	1259	3.3±0.34	CIS-NS4	13	796	2.5±0.28	NS4	1	660	3.9±0.55
CIS-SE5	10	639	3.5±0.81	SE5	1	501	3.5±0.40	CIS-NS5	10	1049	2.6±0.32	NS5	1	209	3.6±0.50
CIS-SE6	10	1435	3.5±0.28	SE6	1	1219	3.5±0.25								
Total	66	5834	3.2±0.47		7	5653	3.5±0.68		53	3858	2.5±0.32		7	2419	3.5±0.76
Chromosome 15															
CIS-SE1	10	688	2.0±0.02	SE1	1	1051	2.0±0.03	CIS-NS1	20	1687	1.8±0.31	NS1	1	184	1.4±0.26
CIS-SE2	17	1413	2.6±0.25	SE2	2	1289	2.7±0.24	CIS-NS2	14	1125	1.9±0.43	NS2	1	238	1.8±0.30
CIS-SE3	18	1423	3.3±0.42	SE3	1	1380	3.3±0.31	CIS-NS3	6	283	2.0±0.03	NS3	3	880	2.0±0.05
CIS-SE4	22	1934	3.3±0.32	SE4	1	1572	3.3±0.24	CIS-NS4	9	490	2.0±0.04	NS4	3	837	2.0±0.06
CIS-SE5	15	1130	3.9±0.22	SE5	1	1377	3.9±0.18	CIS-NS5	18	1561	1.8±0.32	NS5	1	168	1.7±0.23
CIS-SE6	13	1802	2.3±0.19	SE6	1	1487	2.4±0.26								
Total	95	8390	2.9±0.72		7	8156	3.0±0.66		67	5146	1.9±0.11		9	1515	1.8±0.26

Summary of data of numerical analysis of centromeric regions of chromosomes 1, 12 and 15 in six seminomas (SE), five nonseminomatous testicular germ cell tumors (NS), as well as their adjacent CIS-SE and CIS-NS, respectively. Placental like alkaline phosphatase or cytokeratin expression was used as immunohistochemical marker for tumor cells. The number of locations or seminiferous tubules (n^{loc}), cells (n^{cells}), mean number of spots per interphase nucleus (MNSN) and standard deviations are shown.

MATERIAL AND METHODS

Materials

Material of invasive components of six SE and five NS as well as their adjacent parenchyma was snap frozen and studied immunohistochemically on tissue sections for the presence of CIS and different tumor components using PLAP and cytokeratin expression as described before (14). In addition, single parameter DNA flow cytometry was performed to determine the DNA content of the CIS cells (see "Single Parameter DNA Flow Cytometry").

DNA probes and labeling

Chromosome 1, 12 and 15 derived DNA probes, designated pUC1.77 (21), pα12H8 (22) and D15Z1 (23) respectively, hybridizing specifically to repetitive DNA sequences localized in the centromeric regions of these chromosomes under the proper hybridization conditions were used throughout this study. Isolated 1.77-, 1.44- and 1.80-kb in inserts (using standard electro-elution methods) were nick-translated with biotin-11-dUTP using a commercial kit (Bethesda Research Laboratories, Bethesda, Maryland), according to the manufacturer's directions.

Single parameter DNA flow cytometry

DNA flow cytometric analysis was performed as described before using histologically characterized and separately processed CIS and tumor components (24, 38). DNA content was expressed by the DNA Index i.e., the ratio between the modal G0,1 peak of the aneuploid population and that of the modal G0,1 peak of the diploid normal cells in the sample. By definition, a diploid cell population has a DNA index of 1.00. The G0,1 peak could be identified on the basis of its relative position to the trout red blood cell ploidy reference (38, 39).

Simultaneous application of immunohistochemistry and in situ hydridization

Frozen 20-μm tissue sections were mounted on Tissue Adhesive (Abbott Laboratories, Chicago, Illinois) coated slides, air dried overnight, and fixed in 70% ethanol at -20°C for 1 hour. Endogenous peroxidase was inactivated using 0.3% H_2O_2 in methanol for 10 minutes at room temperature. Immunohistochemical staining for PLAP (DakoPatts, Glostrup, Denmark), LCA (DakoPatts), and cytokeratin (CAM 5.2) (Becton Dickinson, San Jose, California) expression was conducted as described (40); dilution of the primary reagent had to be optimized for every specimen examined. Binding of the reagents was visualized by using peroxidase-labeled rabbit-antimouse or swine-anti-rabbit (DakoPatts) second antibody. Protein digestion was performed using pepsine (Sigma, St. Louis, Missouri) (0.0005%, 37°C, for ~5 minutes; this had to be optimized for every specimen examined); dehydration was performed using ethanol in a concentration of 50, 70, and 100%, respectively, and finally the slides were air dried. Rehydration was achieved using 10 μl hybridization solution for each cm^2 {containing: 50% (v/v) formamide; 5% dextran sulphate (molecular weight 500,000, Sigma); 0.5 X Denhardt's solution, 0.1 mM EDTA, 1% sodium dodecyl sulfate, 250 μg/ml single stranded salmon sperm DNA (type 3, Sigma), 1 mM Tris-HCl (pH 7.4), and 0.1 μg/ml labeled DNA probe in 2 x SSC}. Slides were sealed with a coverslip and denaturated in a convection oven for 10 minutes at 80°C. Incubation occurred overnight in a moist chamber at 37°C. Slides were washed using the following posthybridization schedule: 1) three times 50% formamide/2XSSC for 5 minutes each at 37°C; 2) three times 2 X SSC for 5 minutes each at room temperature; 3) Tris buffered saline 5 minutes room temperature. Detection of the hybrids were

visualized by using a peroxidase-labeled streptavidin-biotin complex (DakoPatts, Denmark). Harris' hematoxylin was used for counterstaining.

Interpretation

Screening of the number of spots per interphase nucleus was performed by one individual, whose results were at random verified by another independent observer. The scoring of numbers of spots per interphase nucleus as well the distribution within a certain localization of the two observers was compared and was considered reproducible when the data differed <5%; otherwise the areas were rescreened by both observes.

Evaluation of the immunohistochemistry-ISH results of the CIS and tumor cells was only performed when at least 95% of 300 whole nuclei of normal host cells (stromal-, premeiotic germ cells and/or Sertoli cells, and/or leukocytes) contained two spots for the probes applied or one in the haploid cells belonging to the spermatogenic lineage.

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Chapter IV.

Clonality of Combined Testicular Germ Cell Tumors of Adults.

Laboratory Investigation (in press).

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ABSTRACT

BACKGROUND: Recently we have shown, by combining *In Situ* hybridization and immunohistochemistry, that carcinoma in situ (CIS) adjacent to seminoma (CIS-SE), like SE, usually contains three copies of the centromeric region of chromosome 15 per tumor cell. In contrast CIS adjacent to nonseminomatous testicular germ cell tumors of adults (CIS-NS), as well as NS itself, have two copies.
EXPERIMENTAL DESIGN: In the present study we have used this approach to investigate the clonal origin of the SE and NS components of combined tumors (CTs). We counted the number of copies of chromosome 15 centromeric regions in tumor cell nuclei of the CIS, SE and NS components of nine CTs.
RESULTS: We show that the number of copies of centromeric regions of chromosome 15 in both the SE and the NS component, and the adjacent CIS of the same CT, may be high (SE-pattern) or low (NS-pattern). In two cases the copy numbers were high in the SE component and its adjacent CIS, and low in the NS component and its adjacent CIS.
CONCLUSIONS: The data suggest that in most CTs the SE and the NS components have a monoclonal origin, and that karyotype evolution in CIS and the invasive tumor is very similar.

INTRODUCTION

Little is known about the chromosomal constitution of carcinoma in situ (CIS), the common precursor of all testicular germ cell tumors of adults (TGCTs) (1). Ploidy analysis shows a hypertriploid DNA content of both CIS and seminoma (SE), and a hypotriploid DNA content for nonseminomatous TGCTs (NS) (2-5). In addition the chromosomal marker i(12p), specific for SE and NS (6), is also found in CIS (7). Cytogenetic analysis has demonstrated a statistically significant overrepresentation of chromosome 15 in SE compared to NS (8). This difference is also found using *In Situ* hybridization (ISH) combined with immunohistochemistry on tissue sections (9), and Southern analysis (unpublished). The ISH study also shows that the nuclei of an invasive tumor and its adjacent CIS contain similar numbers of centromeric regions of chromosome 15. In particular, it appears that the number of copies in CIS-SE and SE is about three, while the number of copies in CIS-NS and NS is about two. These data suggest a similar karyotype evolution of both CIS and the invasive component of TGCTs.

Combined TGCTs (CT) have both a SE and a NS component (10), and become clinically manifest at an age in between that of NS and SE (5,10). As in their pure counterparts (11-14), CIS is also often demonstrable in the adjacent parenchyma. Theoretically the SE and the NS components within a CT could have independent origins (biclonal). This would result in a heterogeneous constitution of the population of CIS, showing both a SE- and a NS-pattern. Alternatively, the two components of CT have a monoclonal origin, in which case one would expect to find one type of CIS.

We did interphase cytogenetics as described (9), using a chromosome 15 centromeric region specific DNA probe, to study the clonality of CTs.

EXPERIMENTAL DESIGN:

Nine orchidectomy specimens with a CT were included in this study. Snap frozen material of both the SE and NS components as well as of the adjacent parenchyma were used. Simultaneous application of ISH using a chromosome 15 centromeric specific DNA probe, and immunohistochemistry for the detection of placental like alkaline phosphatase or cytokeratins, was performed as described (9). In total 21,761 nuclei (median: 515

nuclei per area [typically an area consists of two to three adjacent low power fields]; range: 30-2662), in 39 different samples of the different components within the nine cases, were scored for the number of signals. For each tumor specimen it was tried to analyze the SE component and immediately adjacent CIS (CIS-SE), and the NS component and immediately adjacent CIS (CIS-NS). For SE an area was chosen either of the main mass of this component or an area containing diffusely infiltrating SE cells (at a distance from but in continuity with the main mass) between seminiferous tubules, which often contained CIS. For statistical analysis Student's t-test was used.

RESULTS AND DISCUSSION

The question whether SE and NS are derived from a single CIS, and therefore monoclonal, or both have their own precursor is still a matter of debate (5,8,15-18). The CT, showing both histological tumor types within one specimen, accounting for approximately 10% of all TGCTs (10), offer an opportunity to address this problem. Ploidy analysis of separate SE and NS components of CTs shows a hypertriploid DNA content for SE and a hypotriploid DNA content for NS similar to their pure counterparts (3-5), which supports the idea that they follow a similar evolution. Independent of the histology of the invasive tumor, CIS shows a hypertriploid DNA content (2,3). Recently we verified the cytogenetic finding of an overrepresentation of chromosome 15 in SE compared to NS (8), by ISH on tissue sections (9), showing 3.0($\pm$0.7) copies for SE, 2.9($\pm$0.7) for CIS-SE, 1.8($\pm$0.3) for NS, and 1.9($\pm$0.1) for CIS-NS. Apparently, the invasive component and its adjacent CIS show a similar numerical distribution of chromosome 15 centromeric regions. It is noteworthy that CIS-SE and CIS-NS have the same ploidy (2,3), and yet show this consistent difference in number of copies of centromeric regions of chromosome 15. Probably in CIS-NS other chromosomes are present in higher numbers resulting in the same DNA content. Here, the number of copies of chromosome 15 is used as a "marker" to study the relationship between CIS and the SE and NS components in a series of nine CTs.

Some pathology data are summarized in Table I.

Table I.
Pathology of nine combined tumors of the testis

Case#	Age	Size		Histological Components
		SE	NS	
1	34	L	S	SE(+TrGC);EC;EB;TI;TrGc;CIS
2	28	S	L	SE;EC;TI;TD;YO;CIS
3	25	S	L	SE;EC;TI;YO;TrGC;CIS
4	34	S	L	SE;EC;CIS
5	28	L	L	SE;EC;YO;CIS
6	29	L	L	SE;EC;CIS
7	26	L	L	SE;EC;TD;YO;CH;CIS
8	37	S	L	SE;EC;TD;YO;CIS
9	26	L	L	SE;EC;TI;TD;YO;TrGC;CIS

L=Large (largest diameter>0,5cm); S=small (largest diameter<0,5cm); SE=seminoma; NS=nonseminomatous germ cell tumor; TrGC=trophoblastic giant cells; EC= embryonal carcinoma; EB= embryoid body; CIS= carcinoma in situ; TI= teratoma immature; TD= teratoma differentiated; YO= yolk sac tumor; CH= choriocarcinoma.

The mean age of the patients, about 30 years, is in agreement with earlier reports (5,10), being in between that of patients with a NS and a SE. In all cases the SE and NS components were geographically separate. In four cases the SE was small (about 0,5 cm in diameter to microscopic) compared to the NS component, and sometimes not grossly detected, but only after microscopic examination. In one case the NS component was the smallest of the two. The NS always contained an embryonal carcinoma (EC) component. In one tumor this was the only NS element, the other tumors had a mixed histology. Five tumors had a teratoma (TE) and one a choriocarcinoma (CH) component.

The results of the in situ numerical analysis of centromeric regions of chromosome 15 are shown in Table II, and illustrated in Figure 1 (color plate). Due to the thickness of the tissue sections used (20 micron), not all signals are in focus at the same time. In all cases it was possible to analyze the invasive SE and NS components. In three cases both diffusely infiltrating SE cells and SE cells from the main tumor mass were analyzed. For the NS component the EC component was always analyzed. In the five cases which had a TE component (immature or differentiated) this was also analyzed. Only two cases allowed the study of both the CIS-SE and CIS-NS components. In five cases only the CIS-SE, in one case only the CIS-NS, and in one case only CIS, which could not be associated to the SE or the NS component, was analyzed. In two of the five cases where only CIS-SE was analyzed another area of CIS not associated to SE or NS was also included.

The frequency distribution of the signals per interphase nucleus was consistent within one specimen, but varied between specimens, in agreement with our previous findings (9). The exceptions in this regard were the SE and NS components of cases 5 and 9, and the EC and TE components of cases 2, 3 and 8. The number of copies does not seem to correlate with the patients age, or the size of the SE or NS component. Both factors could be related to more advanced tumor progression. When the data from the 39 samples are lumped together the mean number of copies of chromosome 15 centromeric regions in CT is 2.5 ± 0.5. That is in between the number of copies per tumor cell nucleus in SE and in NS. When the data of the different components are put together we find similar results: all components have mean values of about 2.5. The only significant difference appears when all invasive SE components (diSE and SE from Table II) are compared with all invasive NS components, with mean values of 2.7 ± 0.5 and 2.3 ± 0.5 respectively ($p < 0.02$). This result is in agreement with data reported earlier (8,9). The unexpected finding of a lower number of signals in the TE than in the EC samples of the NS component in three cases (case 2,3 and 8) is in agreement with the finding that in karyotypes a slightly lower number of copies of chromosome 15 is found in residual mature teratoma than in primary NS (8,19,20). More cases are needed to confirm these data.

The numbers of copies of chromosome 15 in CTs appear in three patterns: Cluster 1, four cases (3,4,7 and 8) shows a low mean number of signals per nucleus (MNSN) in all the analyzed components (< 2.5, mean 2.2 ± 0.2); Cluster 2, three cases (1,2 and 6) shows a high MNSN (≥ 2.5, mean 3.0 ± 0.3); Cluster 3, two cases (5 and 9), has a high MNSN in the SE and CIS-SE (≥ 2.5, mean 2.7 ± 0.1) and a low MNSN in the NS and CIS-NS components (< 2.5, mean 2.0 ± 0.1). Within the clusters 1 and 2 there were no statistically significant differences between CIS-SE, SE, CIS-NS, EC, and TE. Within cluster 3 the difference between SE and CIS-SE on the one hand and NS and CIS-NS on the other was highly significant ($p < 0.001$). There was no difference between cluster 1 and the NS part of cluster 3, and also no difference between cluster 2 and the SE part of cluster 3.

Figure 2 shows how we envisage that the number of copies of chromosome 15 may change during tumor progression, starting with 4 copies in an originally tetraploid CIS

cell. Obviously different scenarios are conceivable. For example the result in group 3 (E in the figure), would also be found when independently the scenarios A and B took place at the same time. We prefer scenario E, however, because in the few cases where the two components of a CT were karyotyped, these usually had common chromosomal rearrangements (8). The finding of SE and CIS-SE with low copy numbers of chromosome 15 in scenario D, could be due to persistence of the SE phenotype, which in pure NS is more or less immediately lost when the CIS cells become invasive (21). The situation of NS and CIS-NS with high copy numbers in scenario B, may reflect the situation that an invasive SE progresses to a NS. The existence of this pathway gains broader acceptance recently (22) and was proposed by Oliver (23) and us (5), among others, on the basis of ploidy evolution in TGCTs.

Apparently in CTs the CIS cells when they become invasive may develop as both SE and NS. In pure SE and pure NS CIS develops as either SE or NS. This may be reflected by the copy number of chromosome 15, which on average is 2.5, that is in between that of SE and NS. Obviously in CIS the SE phenotype is compatible with a low copy number of chromosome 15 and the NS phenotype with a high copy number of it. This does not come as a surprise since the numbers we found in our previous paper (9) were averages with a rather broad range. Also, biologically it is hard to imagine that the expression of the SE or NS phenotype would depend on the number of copies of one chromosome. This rather depends on cross talk between several genes some of which may be on chromosome 15.

Scenario	A	B	C	D	E
Preclinical stage	CIS (4)* ↓	CIS (4) ↓	CIS (4) ↓ CIS (3) ↓	CIS (4) ↓ CIS (3) ↓	CIS (4) ↓
Clinically manifest tumor	CIS (3) → SE (3)	CIS (3) → SE (3) → NS (3)	CIS (2) → NS (2)	CIS (2) → SE (2) → NS (2)	CIS (3) → SE (3) ↓ CIS (2) → NS (2)
Comment	pure SE: CIS cell maintains phenotype when becoming invasive	monoclonal CT with high copy number: evolution of NS from invasive SE component (group 2)	Pure NS: CIS cell looses SE phenotype more or less immediately when becoming invasive	monoclonal CT with low copy number: evolution of NS from invasive SE component? (group 1)	biclonal CT with high and low copy number: (group 3)

CIS = Carcinoma in situ; SE = seminoma; NS = nonseminomatous germ cell tumor; CT = combined tumor; * figures in brackets indicate the number of copies of chromosome 15.

Figure 2: Models of clonal tumor progression to explain the numbers of copies (figures in brackets) of chromosome 15, starting with tetraploid CIS with 4 copies: pure SE (A), pure NS (C), CT with high copy number of chromosome 15 (B), CT with low copy number of chromosome 15 (D), and CT with high and low copy number of chromosome 15 (E).

METHODS

TUMOR MATERIAL

The series consisted of 9 orchidectomy specimens with a primary NS with among other components a SE component, and therefore, CT, according to the British Classification (10). The tumors were classified according to the WHO-classification (24), on the basis of haematoxylin and eosin stained paraffin embedded tissue sections (Table I). If necessary, the histological findings were supported by immunohistochemistry as

described (5). Cytokeratin expression pattern, for example, may help to distinguish between EC and a solid YS component.

In Situ HYBRIDIZATION AND IMMUNOHISTOCHEMISTRY

The probe pHSr, locus D15Z1 (25) was used, which under proper hybridization conditions specifically recognizes repetitive DNA sequences localized in the centromeric regions of chromosome 15. Frozen tissue sections of 20 micron thickness were treated as reported (9). Briefly, the biotin labeled probe was dissolved in hybridization solution to a final concentration of 10 ng DNA per microliter. After a mild proteolytic digestion of the tissue section, the probe and target DNA were simultaneously denaturated in a 80°C convection oven for 10 minutes and hybridized overnight in a moist chamber at 37°C. After post-hybridization washes the hybrids were visualized by a peroxidase-labeled streptavidin-biotin complex. Haematoxylin was used for counterstaining. The reliability of this procedure, as well as the scoring and interpretation, were reported previously (9).

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TABLE II
Number of copies of chromosome 15 centromeric regions
(mean number of spots/nucleus [X], and standard deviation [SD]) in
different histological components of nine combined tumors of the testis

Case #	HISTOLOGY													
	CISnos		CIS-SE		diSE		SE		CIS-NS		EC		TE	
	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD
1	-		3.4*	0.7	3.4	0.7	3.6	0.7	-		3.0	0.8	2.9	0.8
2	-		-		-		3.4	0.9	3.0	0.7	3.1	0.6	2.6	0.7
3	2.1	0.7	-		-		2.2	0.7	-		2.2	0.7	1.6	0.6
4	-		2.1*	0.5	2.1	0.6	-		-		1.9	0.6	-	
5	-		2.6	0.7	-		2.5	0.7	2.1	0.6	2.0	0.6	-	
6	2.7	0.5	2.7	0.5	-		2.8	0.5	-		2.6	0.6	-	
7	2.4	0.5	2.3*	0.6	2.4	0.6	2.3	0.6	-		2.2	0.6	2.2	0.5
8	-		2.3*	0.6	2.3	0.6	-		2.3	0.6	2.2	0.7	1.5	0.5
9	-		2.7*	0.6	2.7	0.6	2.8	0.6	-		2.0**	0.6	-	
Total:	2.4	0.3	2.6	0.4	2.6	0.5	2.8	0.5	2.5	0.5	2.4	0.4	2.2	0.6

Abbr.: CISnos = carcinoma in situ, not otherwise specified, i.e. not associated to a the SE or the NS component; CIS-SE = CIS adjacent to seminoma; CIS-NS = CIS adjacent to nonseminoma; diSE = diffusely infiltrating seminoma apart from the main mass of the tumor into the surrounding parenchyma; SE = seminoma; EC = embryonal carcinoma; T = teratoma; - = not available.
* CIS adjacent to diffusely infiltrating seminoma; ** EC mixed with yolk sac tumor and immature teratoma.

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Chapter V.

Testicular Germ Cell Tumors of Adults Show Deletions of Chromosomal Bands 11p13 and 11p15.5, but No Abnormalities Within the Zinc-Finger Regions and Exons 2 and 6 of the Wilms' Tumor I Gene.

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ABSTRACT

We have studied the involvement of chromosomal bands 11p13 and 11p15.5 in 15 testicular seminomas (SE) and 18 testicular nonseminomatous germ cell tumors (NS). No allelic imbalances were found in 40% of the SE and 44% of the NS. Loss of heterozygosity (LOH) at 11p15.5 was seen in 21% of the SE and 47% of the NS; the corresponding frequencies for 11p13 were 47% and 44%. Both regions were deleted in 13% of the SE and 44% of the NS, indicating that all NS with a complete LOH of 11p13 also lost the 11p15.5 region. In one (out of two) SE and in five (out of eight) NS, this was due to at least two separate deletions. Loss of the whole p-arm was likely in one SE and two NS. No gross genomic changes of the Wilms' tumor I (*WT1*) tumor suppressor gene were found using a cDNA probe (WT33). Nor were aberrations found in the zinc-finger regions and exons 2 and 6 of this gene, using polymerase chain reaction amplification, single stranded DNA polymorphism analysis, and sequencing. We suggest that loss of genetic information from the short arm of chromosome 11, without affecting the *WT*1 gene in the regions studied, is relatively frequent but not crucial in the pathogenesis of testicular germ cell tumors of adults.

INTRODUCTION

Testicular germ cell tumors of adults (TGCTs) show a consistent peritriploid DNA content (Oosterhuis et al., 1989; Fosså et al., 1991a, b; El Naggar et al., 1992) with a more or less specific chromosomal distribution (De Jong et al., 1990). Loss of parts of or all of chromosomes 4, 5, 10, 11, 13, and 18 is common. Loss of heterozygosity (LOH) from several of these chromosomal regions has been reported (Parrington et al., 1987; Lothe et al., 1989; Radice et al., 1989; Rukstalis et al., 1989; Peltomäki et al., 1990; Murty et al., 1992), and the position here of tumor suppressor gene(s) important in the pathogenesis of TGCTs is imaginable (Marshall, 1991).

Involvement of the short arm of chromosome 11 has been described in a variety of tumors (Slater and Mannens, 1992; Vandamme et al., 1992; Viel et al., 1992), including TGCTs (Lothe et al., 1989; Radice et al., 1989). These two studies reported LOH from 11p15.5 in about 25% of the TGCTs. Besides this band, 11p13 is also reported to be involved in different tumors (Slater and Mannens, 1992; Vandamme et al., 1992; Viel et al., 1992). This is especially interesting because the recently isolated and characterized Wilms' tumor 1 (*WT1*) gene maps here (Bonetta et al., 1990; Call et al., 1990; Gessler et al., 1990). The detailed genomic organization of this gene has been described (Tadokoro et al., 1992b). Besides its role in the development of sporadic Wilms' tumors (Slater and Mannens, 1992), this tumor suppressor gene is suggested to play a role in the development of the mouse urogenital system (Pelletier et al., 1991a,b,c; Amstrong et al., 1992; Bruening et al., 1992; Pritchard-Jones et al., 1992). Its putative role in the development of the human gonadal system is illustrated by the abnormalities of these organs in Denys DRASH and WAGR patients; this is associated with aberration of the zinc-finger regions of the *WT1* gene (Van Heyningen et al., 1990; Pelletier et al., 1991a; Baird et al., 1992; Brown et al., 1992; Coppes et al., 1992; Slater and Mannens, 1992;Akasaka et al., 1993; Ogawa et al., 1993). In sporadic Wilms' tumors, inactivation of this gene can also be due to deletions affecting the zinc-finger regions (Haber et al., 1990; Cowell et al., 1991; Huff et al., 1991; Pelletier et al., 1991a; Ton et al., 1991; Kikuchi et al., 1992; Slater and Mannens, 1992; Tadokora et al., 1992a).

Against this background we decided to study TGCTs for the presence of genomic abnormalities in 11p13 and 11p15.5.

MATERIALS AND METHODS

Samples

Tumor specimens were collected in The Netherlands from 33 patients with a primary germ cell tumor of the testis. The tumors were classified according to the WHO-classification (Mostofi and Sobin, 1977; Mostofi et al., 1987) on the basis of representative hematoxylin and eosin stained sections of frozen and paraffin embedded tissue. If necessary, the diagnosis was supported by immunohistochemistry (Oosterhuis et al., 1989). For isolation of control DNA, after informed consent heparinized peripheral blood was collected from the patients.

Southern Blot Analysis

High molecular-weight DNA was extracted from each sample using standard procedures (sodium dodecyl sulphate-proteinase K and phenol-chloroform extraction and ethanol precipitation) (Maniatis et al., 1982). All tumor samples contained at least 60% tumor cells, as analyzed by light microscopy. Ten micrograms of DNA was digested to completion with the appropriate restriction endonucleases according to the manufacturer's prescription. Fragments were electrophoresed on a 0.8% agarose gel with a Tris-acetate buffer, and transferred to nylon membrane (Hybond N+; Amersham, Inc., Arlington Heights, IL) according to Southern (1975). DNA loading variation was checked by visual judgement of ethidium bromide stained gels. In addition, probe IGH (Croce et al., 1979) was used as reference of loading variation and LOH. This probe did not show LOH in a large study of TGCTs, including this series (Looijenga et al., in preparation). After hybridization, the filters were washed (two times 2xSSC/0.1% SDS, two times 1xSSC/0.1% SDS, two times 0.3xSSC/0.1% SDS, two times 0.1xSSC/0.1% SDS, respectively, at 65°C) and then left to expose to HPX-44 Medical X-Ray film (Valca, Spain) for 1-5 days at -70°C. Filters were rehybridized with a different DNA probe after the previous hybridization signals had been stripped off. Allelic imbalances were analyzed by densitometric screening (Biorad, Richmond, CA), subtracting the background signal. Increase in signal intensity by more than 25% was scored as overrepresentation. Signal intensity reduction of an allele by more than 30% and less than 50% was scored as partial LOH, while signal intensity reduction of more than 50% was scored as complete LOH.

Probes

Seven polymorphic genomic probes localized to 11p13 were used: i.e., JA81BEO, locus D11S417 (Compton et al., 1988); p5S1.6, locus D11S324; p8B1.25, locus D11S325 (Compton et al., 1988); p56H2.4, locus D11S151 (Huff et al., 1987, 1988); p32-1, locus D11S16 (Feder et al., 1985; Kidd et al., 1987); *FSHB* (Watkins et al., 1985). If possible, different restriction endonuclease digestions were used for each probe. In addition, the cDNA probe WT33 (Call et al., 1990) was used on *Eco*RI and *Taq*I digested DNA samples. Allelic imbalances on 11p15.5 were studied using probe pbc-N1, locus HRAS1 (McBride et al., 1982).

The probes were radioactively (^{32}P-dATP; Amersham) labeled using the random primer synthesis according to the Boehringer kit procedure (Boehringer, Mannheim, Indiapolis, IN). Further information about the probes can be found in the HGM 11 (1991) report.

Polymerase Chain Reaction (PCR) of the *WT1* Zinc-Finger Regions and Exons 2 and 6

Amplification by PCR of the zinc-finger regions and exons 2 and 6 of the *WT1* gene was carried out as described (Haber et al., 1990). In a total volume of 50 μl, 0.5 μg

genomic DNA was amplified in a buffer containing 10 mM Tris, pH 8.8 (at 25°C), 50 mM potassium chloride, 15 mM magnesium chloride, 0.01% Triton X-100, 200 μM each of dATP, dCTP, dGTP, and dTTP, 1 μM of each primer, and 1 unit of Taq polymerase (Promega, Madison, WI). Thirty-one cycles of PCR were carried out using a Perkin-Elmer Cetus PCR Instrument (Perkin-Elmer, Norwalk, CT). The program consisted of: 3 min at 94°C; 1 min, 30 sec at 55°C; 1 min at 72°C for cycle 1, followed by 1 minute at 94°C; 1 min, 30 seconds at 55°C and 1 minute at 72°C for the next 30 cycles, with a final extension at 72°C for 3 min. PCR products were analyzed on a 1.5 % agarose gel. For ^{32}P incorporation, 5μCi of α-dATP in addition to the cold dNTPs was added in a total volume of 25 μl of the PCR reaction, after which a new amplification was performed (see above) of 25 cycles.

Single Stranded Conformation Polymorphism (SSCP) and Sequencing

All SSCP experiments were performed with fairly clean amplicons. These were obtained by dilution 100 times in SSCP buffer (0.1%SDS/10/mM EDTA). Of this, 2 μl was added to 2 μl of formamide dye (95% formamide, 20 mM EDTA, 0.05% Bromphenol blue, and 0.05% xylene cyanol). Denaturation was performed at 85°C for 5 min, after which the samples were loaded on a 5% 40:1 bis-acrylamide gel. Electrophoresis was performed at 4°C, 30 Watt, using 1xTBE buffer, pH=8.3. After drying the gel with a vacuum dryer (New Brunswick Scientific, Benelux B.V.), it was left to expose a HPX-44 Medical X-Ray film (Valca, Spain) overnight at -80°C. Samples showing difference in mobility on the SSCP gel were sequenced. PCR products for sequencing were isolated by excision after electrophoresis on a 1% low melting point agarose (Biorad) gel, and wrapped in plastic frozen at -20°C. Next morning, DNA was squeezed out of the gel slize and sequenced (T7 polymerase DNA sequencing kit, Promega). Analysis was performed after electrophoreses using a 6% 6M poly-acrylamide gel at room temperature, after drying of the gel and exposure as described.

RESULTS

The histological tumor classification and the age at diagnosis of the series of 33 patients are shown in Table 1. Examples of Southern blot analysis are illustrated in Figure 1, showing three SE (cases G009, G010, and G011) and four NS (cases G007, G017, G019, and G020).

The results of the presence of aberrations in chromosomal regions 11p13 and 11p15.5 are summarized in Figure 2 and Table 2. The most common abnormality of 11p13 and 11p15.5 was complete LOH. Subband 11p15.5 showed complete LOH in 32% of all TGCTs, with a higher incidence in NS than in SE. In contrast, more SE than NS showed complete LOH at 11p13, accounting for 39% in total. Partial LOH was only detected in NS, in cases G007, G020, and G021 from band 11p13, in case G007 also from 11p15.5. All NS showing a complete 11p13 LOH contained also 11p15.5 LOH (cases G017, G018, G019, G031, G034, and G035). This was found also for two of the three SE (G011 and G012). Two NS showed LOH of all informative probes, indicative of loss of the whole p-arm (G031 and G035). The presence of at least two separate deletions could be identified in three NS (G017, G018, and G019) and one SE (G011). In addition, one NS (G007) showed a similar pattern, but partial LOH was found for both loci. Another NS (G020) showed complete LOH of 11p15.5 and a separate partial LOH at 11p13. No gross DNA rearrangements or deletions of the *WT1* gene were found using the WT33 cDNA probe (not shown).

TABLE 1.

Age of the Patients and Histological Data of the 33 Primary Testicular Germ Cell Tumors.

Case	Age (yrs)	Histology[a]	Components[a]
G001	22	NS	EC,CH,TE (M+I)
02	39	SE	
03	20	NS	EC
04	29	SE	
06	24	NS	EC
07	46	NS	TE(M),EC
08	33	NS	EC,CH,YS,TE(M)
09	45	SE	
10	39	SE	
11	37	SE	
12	47	SE	
13	29	SE	
14	31	SE	
15	19	NS	EC,YS
16	23	NS	EC,TE(M+I),YS
17	43	NS	TE(M),CH
18	16	NS	EC
19	51	NS	EC,YS
20	29	NS	EC
21	22	NS	EC,TE(M+I),CH
22	21	NS	EC,TE(M+I),YS
23	71	SE	
24	30	SE	
25	30	SE	
26	64	NS	EC
28	28	SE	
29	29	SE	
30	46	SE	
31	29	NS	TE(M),EC
32	26	NS	TE(M),CH
33	59	SE	
34	37	NS	EC,TE,YS
35	20	NS	TE(I)

[a] SE = seminoma; NS = nonseminomatous testicular germ cell tumor; YS = yolk sac tumor; CH = choriocarcinoma; TE = teratoma (M = mature; I = immature); EC = embryonal carcinoma.

Fourteen PCR amplified fragments showed possible mobility shifts compared with the control sample by SSCP. These were all sequenced. No abnormalities were found (not shown).

Using the Mann Whitney U test, no significant age differences were present between SE and NS patients with and without tumor specific aberrations (not shown).

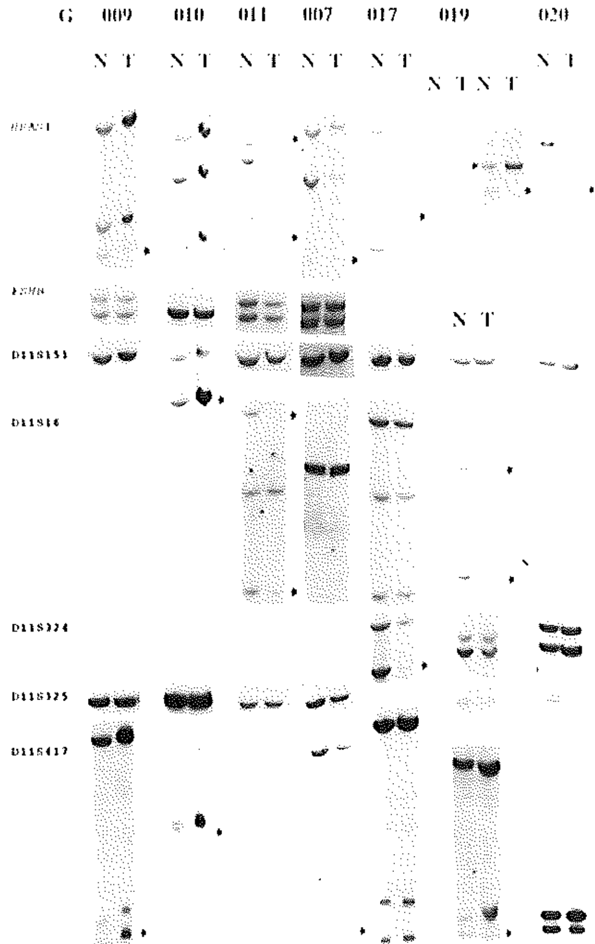

Fig. 1. Examples of Southern blot analysis of allelic imbalances of chromosomal region 11p13 and 11p15.5. Total tumor DNA (T) and normal peripheral blood DNA (N) was digested with the appropriate enzyme, and after electroforesis transferred to nylon membrane and hybridized with multiple probes. Shown are the seminomas G009, G010, and G011, and the NS G007, G017, G019 (two restriction endonuclease digestions for locus HRASI), and G020. The loci are indicated, as well as the imbalances, by asteriks.

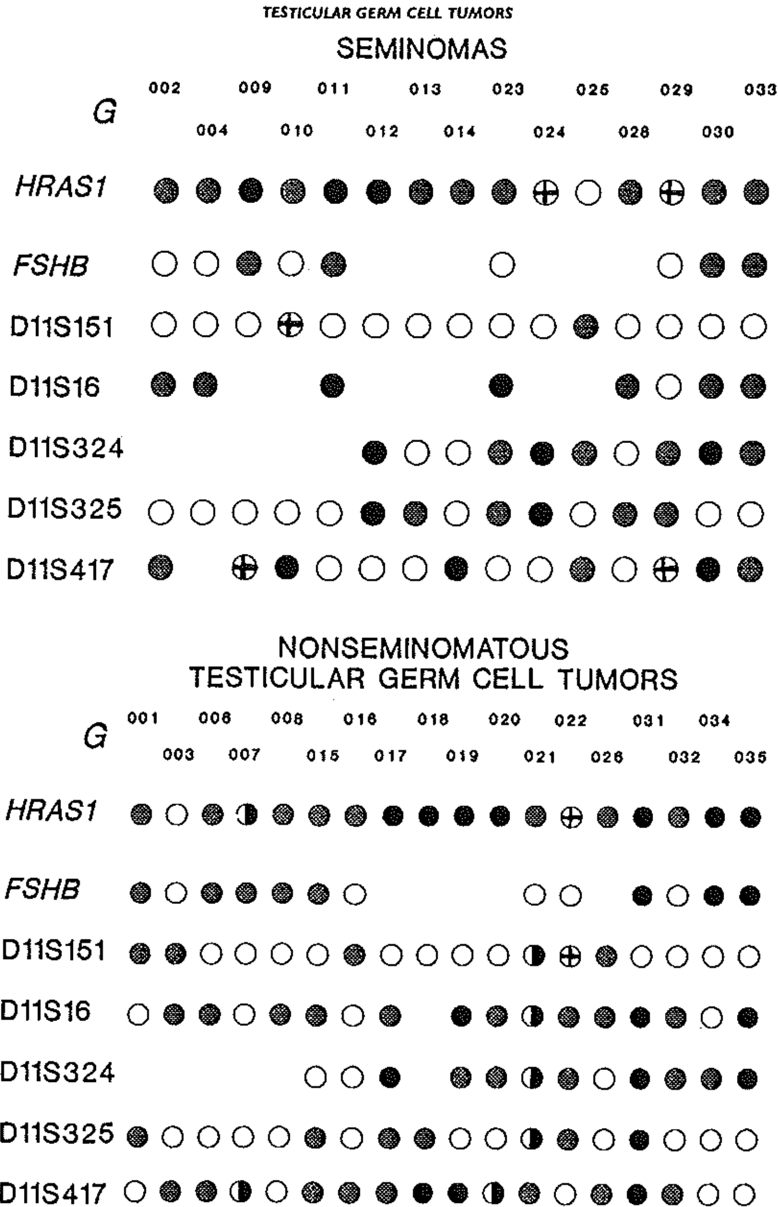

Fig. 2. Summary of the genomic aberrations of chromosomal region 11p13 and 11p15.5 in 33 primary testicular germ cell tumors. ○ = non informative; ◍ = informative; ◑ = partial loss of heterozygosity; ● = complete loss of heterozygosity; ⊕ = overrepresentation.

TABLE 2.
Involvement (%) of Bands 11p13 and 11p15.5 in 33 Primary Testicular Germ Cell Tumors, in Total (T) and Separated as Seminomas (SE) and Nonseminomatous Tumors (NS).

Map Localization	Locus	Informative		LOH Complete			LOH Partial			Overrepresented		
		SE	NS	SE	NS	T	SE	NS	T	SE	NS	T
11p15.5	HRAS	14/15	17/18	21.4	41.2	32.3	0.0	5.9	3.2	14.3	5.9	9.7
11p13	*FSHB*	4/9	8/13	0.0	37.5	25.0	0.0	0.0	0.0	0.0	0.0	0.0
	D11S151	2/15	6/18	0.0	0.0	0.0	0.0	16.8	12.5	50.0	6.7	28.6
	D11S16	7/8	13/17	28.6	23.1	25.0	0.0	7.7	5.0	0.0	0.0	0.0
	D11S324	7/10	9/12	42.9	33.3	37.5	0.0	11.1	6.3	0.0	0.0	0.0
	D11S325	6/15	7/18	33.3	14.3	23.1	0.0	14.3	7.7	0.0	0.0	0.0
	D11S417	8/14	13/18	37.5	23.1	28.6	0.0	15.4	9.5	25.0	0.0	9.5
11p13*				46.7	33.3	39.4	0.0	16.7	9.1	20.0	5.6	12.1
11p13 and 11p15.5		15/15	18/18	13.3	44.4	33.3	0.0	11.1	6.1	6.7	5.6	6.1

*At least one locus informative.

DISCUSSION

Because of the consistent aneuploidy of TGCTs (Oosterhuis 1989; De Jong et al., 1990; Oosterhuis et al., 1990; Fosså et al., 1991a,b; El Naggar et al., 1992; Rodriquez et al., 1992), polyploidization is probably an early event in the pathogenesis. In the following phase of karyotype evolution these tumors develop a more or less specific pattern of overrepresentation and underrepresentation of chromosomes or chromosomal parts (for review see De Jong et al., 1990). Theoretically, assuming that loss of chromosomes is a random process, a triploid cell has a chance of one in three to become hemizygous for a chromosomal region upon further loss of chromosomal material. A higher percentage of loss of heterozygosity (LOH) of a chromosomal region suggests nonrandomness, and would indicate an important, probably early event in the pathogenesis. The various studies of tumor-specific deletions in TGCTs, the finding of which would indicate the presence of tumor suppressor gene(s), do not show a consistent pattern of affected loci (Parrington et al., 1987; Lothe et al., 1989; Radice et al., 1989; Rukstalis et al., 1989; Peltomäki et al., 1990; Murty et al., 1992). The involvement of chromosome 11, one of the underrepresented chromosomes (De Jong et al., 1990), has been studied by several groups. LOH of chromosomal region 11p15.5 has been reported in 26% of 31 TGCTs and in 29% of 14 TGCTs by Lothe et al. (1989) and Radice et al. (1989), respectively. The first report showed LOH in the same percentage of SE and NS, while the second showed more LOH in NS than in SE. In contrast, no involvement of 11p15.5 was found in another series, using partially the same probes (Peltomäki et al., 1990). We found complete LOH from 11p15.5 using the same probe in 21% of the SE and 41% of the NS, adding up to a total of 32%. The higher number of NS than SE with 11p15.5 changes reported by Radice et al. (1989) is corroborated by our data. This could be due to the lower copy number of chromosome 11 in NS compared to SE, as assessed by karyotyping (De Jong et al., 1990).

Deletions of 11p13 have also been described in different tumors (Vandamme et al., 1992; Veil et al., 1992). For example, in ovarian carcinomas this band is a deletion hot spot, and changes here correlate with clinical behavior (Viel et al., 1992). Another example is Wilms' tumor (for review see Slater and Mannens, 1992), of which the non-familial type is correlated with 11p13 deletions. From this band, a tumor suppressor gene called *WT1* was recently isolated and characterized (Bonetta et al., 1990; Call et al., 1990; Gessler et al., 1990). Its involvement in Wilms' tumor development was subsequently reported (Brown et al., 1992; Kikuchi et al., 1992; Slater and Mannens, 1992; Tadokora et al., 1992a). Results of Nothern blotting and mRNA in situ hybridization suggest that the *WT1* gene plays a role in the development of the murine urogenital system, including testis, ovary and uterus (Pelletier et al., 1991c; Amstrong et al., 1992; Prichard-Jones et al., 1992). Its function in the development of the gonads ties this gene to the development of germ cells. This is also illustrated by the possible association of Wilms' tumors and genitourinary abnormalities (Brown et al., 1992; Coppes et al., 1992), like cryptorchism, a risk factor for developing TGCTs (Giwercman et al., 1989). Therefore this gene could be involved in the pathogenesis of TGCTs.

Our series of 33 TGCTs did show indeed deletions of the 11p13 region, i.e., complete LOH of 47% among the SE and 33% among the NS. Combined 11p13 and 11p15.5 deletions (complete or partial) were found in 13% of the SE and 44% of the NS; they were due to two separate deletions in one out two SE and in 63% of the NS. Loss of the whole p-arm was likely in one SE and in two NS. This is in agreement with the lower copy numbers of chromosome 11 in NS then in SE (De Jong et al., 1990), supported by the 11p-overrepresentation mainly in SE and partial LOH only in NS.

The data on complete LOH from 11p13 are not in agreement with this model, because

LOH is found more frequently in SE than in NS. Theoretically, these deletions could occur relatively late in tumor evolution, which is slower in SE than in NS (the median age of the patients was 31 years for SE and 25 years for NS). It must be kept in mind that in aneuploid tumors, like SE and NS, two different mechanisms may cause LOH: (1) loss of whole chromosomes during karyotype evolution, and (2) structural changes giving rise to deletions. Based on the physical localization of the markers, structural rearrangements of the short arm of chromosome 11 are suggested by the molecular data in, e.g., cases G009, G010, G020 and G024. This is in agreement with recent data on chromosomal breakpoints in TGCTs (Van Echten et al., unpublished).

In Wilms' tumors, inactivation of the retained *WT1* tumor suppressor gene is mainly due to aberrations in the zinc-finger encoding regions (Haber et al., 1990; Cowell et al., 1991; Huff et al., 1991; Pelletier et al., 1991a; Ton et al., 1991; Brown et al., 1992; Kikuchi et al., 1992; Slater and Mannens, 1992; Tadokora et al., 1992a; Akasaka et al., 1993). No gross genomic aberrations were found in this series of TGCTs using the cDNA probe WT33. In addition, irrespective of the status of LOH of 11p13 (and 11p15.5), no abnormalities within zinc-finger regions 1 through 4 as well as exons 2 and 6 of the *WT1* were found in either tumor types. This indicates that abnormalities within these regions do not occur during the development of these tumors.

We conclude that 11p13 and 11p15.5 abnormalities occur relatively frequently, without affecting the zinc-finger regions and exons 2 and 6 of the *WT1* tumor suppressor gene. The percentages are in concordance with random loss in a peritriploid cell. Therefore, they are less significant in the pathogenesis of germ cell tumors of the human testis.

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Chapter VI.

Involvement of Chromosomes 12 and 22 in Testicular Germ Cell Tumors of Adults.

Genes, Chromosomes & Cancer
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ABSTRACT

Loss and/or gain of genomic sequences on chromosomes 12 and 22 was studied in primary human testicular germ cell tumors of adults (TGCTs). Within a series of 15 seminomas (SE) and 17 nonseminomatous TGCTs (NS) no loss of heterozygosity (LOH) of the short arm of chromosome 12 was observed, but overrepresentation was present in 53% of the SE and 88% of the NS. A uniparental origin could be determined in most cases (five out of eight and nine out of 15, respectively). Deletion of band 12q13 or 12q23 was found in 21% of the tumors (three out of 11 SE and two out of 13 NS). Cytogenetic data, available of 24 primary SE and 61 primary NS (of which four SE and 11 NS were included in this study) supported the molecular findings of a high frequency of overrepresentation of the short arm and a low incidence of deletions affecting the long arm of chromosome 12 in primary TGCTs.

In a series of 21 SE and 24 NS, including the tumors studied for chromosome 12, allelic imbalances of chromosome 22 were found in 62% of the SE and in 54% of the NS. While cytogenetic data of TGCTs, including six SE and 15 NS from this study, showed a relatively overrepresentation of chromosome 22 in SE and underrepresentation in NS, molecular analyses showed overrepresentation of the whole chromosome mainly in NS, and loss mainly in SE. This was verified using *in situ* hybridization on thick tissue sections. This discrepancy between the *in vivo* situation and the karyotypic data might be due to the applied isolation technique of the metaphases.

Because clinical presentation of SE with overrepresentation of chromosome 22 sequences, determined by molecular analysis, is at a significantly younger age ($p<0.025$) than of SE without (mean 29 versus 39 years of age), a gene encoding a tumor promoting factor localized on this chromosome is suggested.

In contrast to chromosome 12, a preferential overrepresentation of the maternal chromosome 22 was found in TGCTs, which might be indicative for genomic imprinting.

INTRODUCTION

Testicular germ cell tumors of adults (TGCTs), both seminomas (SE) and nonseminomatous TGCTs (NS), as well as their common precursor, carcinoma in situ (CIS) (Skakkebæk et al., 1987), are consistently aneuploid (Müller et al., 1981; Oosterhuis et al., 1989; De Jong et al., 1990; Vos et al., 1990; Fosså et al., 1991a,b; El-Naggar et al., 1992; De Graaff et al., 1992; Rodriquez et al., 1993). Therefore, aneuploidization is suggested as an early, and possibly crucial, event in the initiation and/or promotion of these tumors (Oosterhuis and Looijenga, 1993). Progression of the tumor in its pre-invasive and invasive stages appears to be associated with net loss of (parts) of chromosomes (Oosterhuis et al., 1989; De Jong et al., 1990; Fosså et al., 1991b; El Naggar et al., 1992). Cytogenetic analyses have shown that certain (parts of) chromosomes are more prone to loss than others (De Jong et al., 1990; Looijenga et al., 1993). Therefore, not only loss of heterozygosity (LOH), indicative for the involvement of tumor suppressor genes, but also overrepresentation of certain chromosomal regions, indicative for the involvement of genes encoding tumor promoting factors may be important in the pathogenesis of TGCTs.

Besides the presence, also the parental origin of certain (parts of) chromosomes appears to be crucial for normal development (De Groot and Hochberg, 1993 for review). This phenomenon has been named genomic imprinting. It is tempting to speculate that in TGCTs, which in their developmental potential mimic early embryonic development, genomic imprinting may play a role in the control of differentiation. This hypothesis can be tested by studying the parental origin of chromosomal regions showing allelic

imbalances, as recently performed for the short arm of chromosome 11 (Lothe et al., 1993).

Here, we assayed a series of primary TGCTs, comprising SE and NS, for allelic imbalances on chromosomes 12 and 22, as well as the parental origin of the gained or lost genomic material in a subgroup of these tumors. Chromosome 12 was studied because of a consistent overrepresentation of the short arm (De Jong et al., 1990; Peltomäki et al., 1992; Geurts van Kessel et al., 1993; Rodriquez et al., 1993), as well as the suggested role of deletions of two loci on the long arm (Murty et al., 1992). Besides the general lack of knowledge about the involvement of chromosome 22 in TGCTs (Radice et al., 1989; Peltomäki et al., 1990), this chromosome was also selected because of the cytogenetical finding of overrepresentation in SE as compared to NS (De Jong et al., 1990). This suggests a relationship between the copy number of this chromosome and the histology of the TGCT.

MATERIALS AND METHODS

Samples

Forty-five fresh, primary unilateral TGCT specimens were collected in the Netherlands. They were diagnosed according to the WHO-classification (Mostofi et al., 1987) using representative frozen and paraffin embedded tissue sections, stained with hematoxylin and eosin. If necessary, the diagnosis was supported by immunohistochemistry as described (Oosterhuis et al., 1989). The age of the patients at diagnosis, as well as the histology of the tumors are summarized in Table 1. After informed consent, in addition to heparinized peripheral blood of the patients themselves as control DNA, peripheral blood was collected from the parents of 10 patients (cases G001-009, and G011).

Cytogenetic analysis

All primary TGCTs were handled for karyotyping as described (Castedo et al., 1989a; Castedo et al., 1989b). Briefly, representative samples of the NS were minced and disaggregated with collagenase D (1 mgram/ml, Boehringer Mannheim) overnight at 34°C. Subsequently the cells were seeded in extracellular matrix coated T75 flasks (Eldan, Israel) and incubated at 34°C in a humidified atmosphere with 5% CO_2 in air. Within four days the cells were exposed for 5 hours to colcemid (0.05μg/ml) and subsequently harvested. In contrast, SE were analyzed after direct harvesting of metaphases from the tumor. The karyotype was described according to the ISCN 1991 recommendations (Mitelman, 1991), except that it is consistently compared to a triploid constitution.

In situ hybridization

In situ hybridization and the interpretation was performed as described (Looijenga et al., 1993). A chromosome 22 centromere specific repetitive DNA probe (D22Z3) was used (McDermid et al., 1986), and at least 100 tumor cells were counted.

Southern blot analysis

High molecular-weight DNA was extracted from each sample using standard procedures (sodium dodecyl sulfate and proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation (Maniatis et al., 1982)). Light microscopically all tumor samples were ascertained to contain a majority of tumor cells. Ten micrograms of DNA were digested to completion using appropriate restriction endonucleases, electrophoresed on a 0.8% agarose gel and transferred to nylon

membranes (Hybond N+; Amersham, Inc, Arlington Heights, Ill) according to Southern (1975). DNA loading variation was checked by visual judgement of ethidium bromide stained gels. In addition, probe IGH (Croce et al.,, 1979) was used as reference for quantitation of loading variation and imbalances. This probe did not show a high frequency of allelic imbalances in a large series of TGCTs, including the cases studied here (unpublished results). After hybridization the filters were washed till 0.1xSSC/0.1% SDS at 65°C and exposed to HPX-44 Medical X-Ray film (Valca, Spain) for 1-5 days at -70°C. Filters were rehybridized with subsequent DNA probes after stripping off the previous hybridization signals. Allelic imbalances were analyzed by densitometric scanning (Biorad, Richmond, CA) and interpreted as described (Looijenga et al., 1993).

TABLE 1.
Age of the Patients and Histological Data of the 45 Primary Testicular Germ Cell Tumors.

Case	Age (yrs)	Histology[a]	Components[b]
G001	22	NS	EC,CH,TE (M+I)
02	39	SE	
03	20	NS	EC
04	29	SE	
06	24	NS	EC
07	46	NS	TE(M),EC
08	33	NS	EC,CH,YS,TE(M)
09	45	SE	
10	39	SE	
11	37	SE	
12	47	SE	
13	29	SE	
14	31	SE	
15	19	NS	EC,YS
16	23	NS	EC,TE(I),MT,YS
17	43	NS	TE(M),CH
19	51	NS	EC,YS
20	29	NS	EC
21	22	NS	EC,TE(M+I),CH
22	21	NS	EC,TE(I),TE(M),YS
23	71	SE	
24	30	SE	
25	30	SE	
26	64	NS	EC
28	28	SE	
29	29	SE	
30	46	SE	
31	29	NS	TE(M),EC
32	26	NS	TE(M),CH
33	59	SE	
34	37	NS	EC,TE(M),YS
35	20	NS	TE(I)
36	36	SE	
37	23	NS	EC,CH
38	53	SE	
39	39	SE	
40	38	SE	
41	21	NS	EC,TE(M),YS
42	31	NS	EC,TE(M)
43	16	NS	EC
44	30	NS	EC
45	28	NS	EC,TE(M),YS
46	23	NS	TE(M),YS
47	25	SE	
48	32	SE	

[a] SE = seminoma; NS = nonseminomatous testicular germ cell tumor.
[b] EC = embryonal carcinoma; CH = choriocarcinoma; YS = yolk sac tumor; TE = teratoma (M=mature; I= immature).

Probes

In this study the following probes [loci] were used; chromosome 12: p12-16 [D12S2], von Willebrand factor 8 [F8VWF], clones p11-1-7 and p11-2-2 [D12S6], p9F11 [D12S4], insulin-like growth factor [IGF1]; chromosome 22: NB17 [D22S181], NB35 [D22S182], NB84 [D22S183], 3'BCR and 5'BCR, NB129 [D22S193], p22/34 [D22S9], NB5 [D22S201], 22C1-18 [D22S10]. The probes were radioactively (^{32}P-dATP; Amersham) labeled using random primer synthesis (Boehringer, Mannheim). Further information about these probes can be found in the HGM 11 (1991) report, and in a recent publication of Mathew et al., (1992).

RESULTS

The results of the Southern blot analyses are illustrated and summarized in Figure 1, Table 2, and Figure 2.

SEMINOMAS

NONSEMINOMATOUS TESTICULAR GERM CELL TUMORS

G D12S2 F8VWF D12S6 D12S4 IGF-1 A.

D22S181 D22S182 D22S183 D22S9 D22S10 D22S193 5'BCR 3'BCR D22S201 B.

Fig. 1. Summary of the Southern blot analysis on chromosomes 12 (A) and 22 (B) in a series of primary testicular seminomas (SE) and primary testicular nonseminomatous germ cell tumors (NS). (○= non informative; ◍ = informative; ◐ = partial loss of heterozygosity; ● = complete loss of heterozygosity; ⊕ = overrepresentation; ⊖ = non informative and overrepresentation; ⊖ = non informative and underrepresentation).

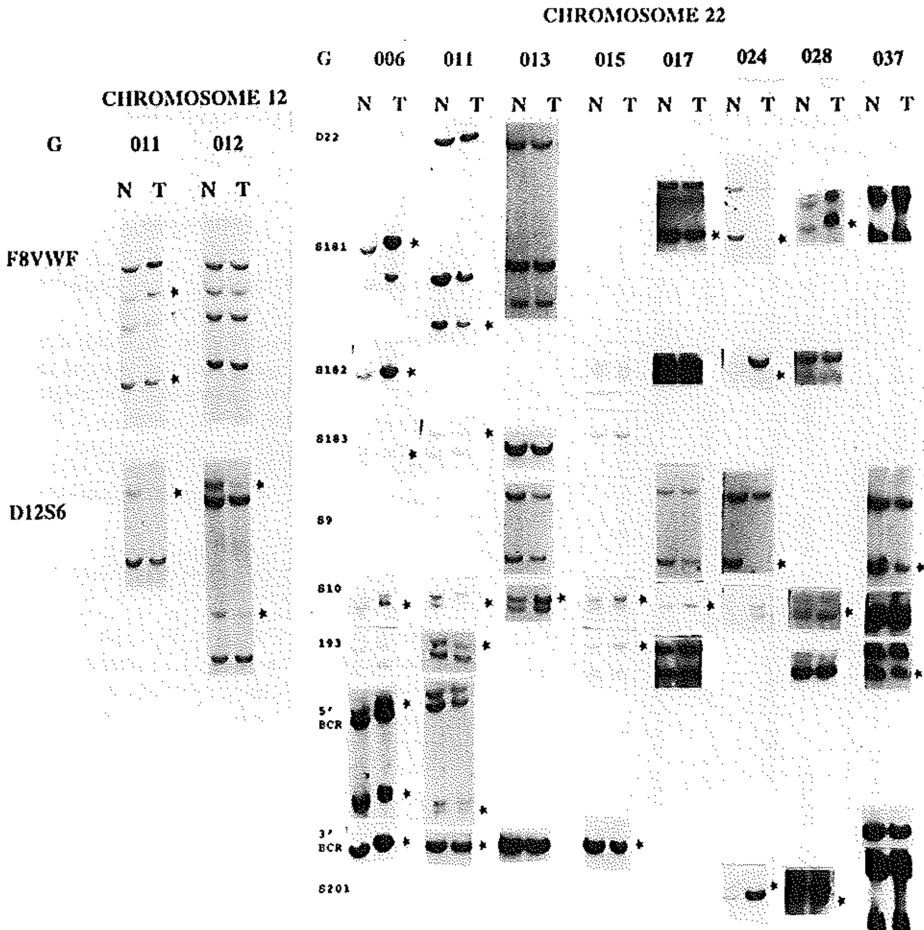


Fig.2. Examples of Southern blot analysis concerning chromosome 12, showing cases G011 and G012; and chromosome 22, showing cases G006, G011, G013, G015, G017, G024, G028 and G037. The probes used, as well as allelic imbalances (asterisk) are indicated.

Table 2.
Percentages of allelic imbalances on chromosome 12 and 22 in the series of seminomas (SE) and nonseminomatous testicular germ cell tumors (NS) studied, in total and separated for the different histologies.

Map localization	Locus	Informative		LOH Compl.			LOH Part.			overrepr.		
		SE	NS	SE	NS	T	SE	NS	T	SE	NS	T
12pter-p12	D12S2	4/9	4/13	0.0	0.0	0.0	0.0	0.0	0.0	50.0	50.0	50.0
	F8VWF	10/14	9/17	0.0	0.0	0.0	0.0	0.0	0.0	40.0	88.9	68.4
total:		12/15	11/17	0.0	0.0	0.0	0.0	0.0	0.0	41.7	81.8	60.9
12q13	D12S6	3/10	7/17	33.3	0.0	10.0	0.0	0.0	0.0	66.7	28.6	40.0
12q23	D12S4	10/15	8/17	20.0	12.5	15.8	0.0	12.5	5.9	0.0	0.0	0.0
	IGF-1	2/9	3/12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	33.3	25.0
22pter-q11	D22S181	13/20	16/24	30.8	12.5	20.7	7.7	18.8	13.8	23.1	18.8	20.7
	D22S182	4/14	7/17	25.0	0.0	8.3	25.0	14.3	18.2	0.0	14.3	9.1
	D22S183	9/24	6/24	44.4	16.7	33.3	0.0	0.0	0.0	44.4	50.0	46.7
22q11.1-q11.2	D22S9	11/21	8/24	27.3	25.0	26.3	18.2	12.5	15.8	9.1	25.0	15.8
22q11.2-qter	D22S10	16/21	16/23	31.3	0.0	15.2	6.3	6.3	6.3	27.3	31.3	38.4
22q11-q12	D22S193	11/21	13/24	18.2	15.4	16.7	18.2	15.4	16.7	27.3	30.8	29.2
22q11	3'BCR			9.5	12.5	8.9				9.5	12.5	11.1
	5'BCR			9.5	4.2	4.4				14.3	16.7	15.5
22q13-qter	D22S201	9/16	10/19	33.3	10.0	21.1	11.1	20.0	15.8	22.2	10.0	15.8

No LOH was found on the short arm of chromosome 12, while uniparental overrepresentation was detected in 61% of the tumors, i.e., 42% of the SE and 82% of the NS. Band 12q13 showed uniparental overrepresentation in two out of three informative SE, and in two out of seven informative NS. LOH was observed in one SE and in none of the NS. Band 12q23 showed LOH in two out of ten informative SE, and two out of eight informative NS. In total 21% of the TGCTs showed LOH of either of these loci on the long arm. Overrepresentation of region 12q23 was found in one NS, which tumor most probably showed overrepresentation of the whole chromosome 12.

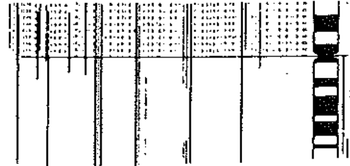
Overrepresentation of probably the entire chromosome 22 was observed in four SE (19%) and five NS (25%). The figures concerning loss of the whole chromosome are 24% and 17%, respectively. Complete deletion affecting regions on chromosome 22 were predominantly found in SE (loci D22S181, D22S182, D22S183, D22S9, D22S10, D22S193, and D22S201), while overrepresentation was mainly present in NS (loci D22S182, D22S183, D22S9, D22S10, D22S193). Chromosomal region 22q11-q12 was involved in 62% of the SE (19% overrepresentation, 38% deletion, 5% both), and in 54% of the NS (29% overrepresentation, 21% deletion, 4% both).

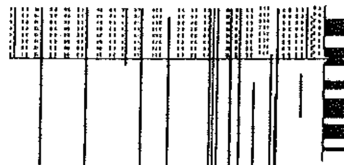
In Figure 3 the relative over- and underrepresentation of (parts of) chromosome 12 (A) and 22 (B), based on karyotypic analysis of 85 primary TGCTs is illustrated (the complete data set will be published elsewhere).

CHROMOSOME 12

SEMINOMA

NONSEMINOMATOUS
TESTICULAR
GERM CELL TUMORS





| = two times |

A.

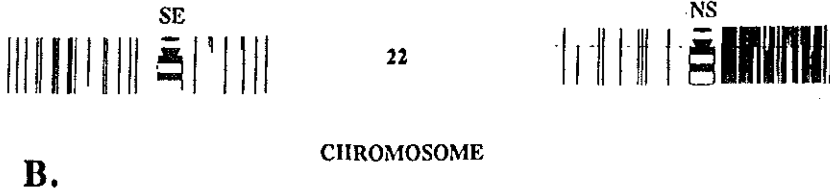


Fig. 3. Schematic presentation of over- and underrepresentation of chromosome 12 and 22 regions based on cytogenetic analysis of 24 primary seminomas (SE) and 61 primary nonseminomatous testicular germ cell tumors (NS). On the left side of the chromosome the relative overrepresentation is indicated, while on the right site the underrepresented regions are indicated (compared to a triploid DNA content, expected number is three).

The karyotypic data concerning chromosome 12 and 22 of the tumors included in this study, i.e., G001, 006-008, 014-016, 017, 019, 021, 022, 024, 026, 029, 032, 036, 041-043, 046, 048, are illustrated in detail in Table 3.

TABLE 3.

Summary of the karyotypic finding on chromosome 12 and 22 in the germ cell tumors of the adult testis also studied using Southern blot analysis.

	chromosome 12			chromosome 22	
	normal	i(12p)	other	normal	other
SE					
014	3	0	dic(7pter-7q32;;?;;12p13-12qter)	5	
024	3	1		2	
029	3	0	dic(1;12)(p13;q22)	2	
032	3	1		2	
036	6	0		2	
048	4	1		4	
NS					
001	3	2		2	
006	3	3		1	
007	3	2		2	
008	2	2	inv(12)(q15;12q24.1)	2	add(22)(22q13)
015	3	2		3	
016	4	4	2 del(12)(12q15-12q24)	3	2 i(22q10)
017	3			3	
019	2			1	
021	3	2	der(10)t(10;12)(10q26;12q13)	2	der(22)t(17;22)(17q11;22p11)
022	2		add(12)(12p13)	2	
026	3		der(14)t(12;14)(12q13;14p11)	1	der(21)t(21;22)(21p11;22q11)
041	3	2		2	
042	2	2	add(12)(q11)?	2	
043	3	2		3	
046	3	3		3	

The molecular data on chromosome 12 are concordant with the chromosomal analysis for both SE and NS (Figure 1 and Table 3). In contrast, some discrepancies are present concerning chromosome 22. While a uniparental overrepresentation of the whole chromosome 22 was found by Southern blot analysis in G029 and 036, chromosomal analysis revealed only two normal copies of this chromosome. In addition, no molecular aberrations on chromosome 22 were detected in G014, while five copies of this chromosome were found by karyotyping. In five out of 15 NS the chromosomal analysis was different from the molecular findings; i.e., two tumors (G006 and 026) showed uniparental overrepresentation while only one chromosome 22 was found, two tumors (G007 and 008) showed uniparental overrepresentation, while only two normal chromosomes were found, and no aberrations were present in G019, while only one normal chromosome 22 was identified by karyotyping. To verify this, ISH was performed on four SE (G014, 024, 032, and 036) and four NS (G006, 007, 015, and 032). The results are summarized in Table 4.

**Table 4.
Summary of the *in situ* hybridization data
on thick tissue sections of
germ cell tumors of the adult testis,
using a chromosome 22 centromere specific DNA probe.**

	case	mean number of spots per tumor nucleus	standard deviation
SE[1]	014	2.0	0.3
	024	2.3	0.6
	032	2.0	0.6
	036	5.0	0.7
NS[2]	006	4.9	0.6
	007	2.1	0.6
	015	1.3	0.4
	043	1.5	0.7

[1]Seminoma; [2]Nonseminomatous testicular germ cell tumor.

For all SE, the ISH findings were in agreement with the molecular data, which was also true for three of the NS. Two of the SE (G014 and 036) showed a discrepancy between the ISH and cytogenetic findings, as well as between the molecular and cytogenetic findings. Three NS (G006, 007, and 043) showed concordant results obtained by ISH and Southern blot analysis. Three SE (G006, 015, and 043) showed a difference between the cytogenetic and ISH data. Two NS (G006 and 007) contained a lower copy number of chromosome 22 by karyotyping than expected from molecular analysis. In summary, seven out of eight tumors (88%) showed ISH data in agreement with molecular analysis, while four out of eight cases (two SE and two NS) showed a discrepancy between the molecular and cytogenetic data.

The parental origin of the alleles showing over- or underrepresentation in tumors G001-G009, and G011 is indicated in Table 5, of which examples are shown in Figure 4.

TABLE 5.
Summary of the parental origin of the short arm of chromosome 12 and chromosome 22 in germ cell tumors of the adult testis.

HISTOLOGY	CASE	12p	22
SE	002	>♂	>♀
	004	>?	>♀
	009	>?	
	011	>♀	>♂*
NS	001	>♂	
	003	>♂	>♂
	006	>♀	>♀
	007	>♂	>♀
	008	>?	>♀

Abbr. SE = seminoma; NS = nonseminomatous testicular germ cell tumor; > = overrepresentation; ♂ = paternal allele; ♀ = maternal allele; * due to loss of the maternal allele.

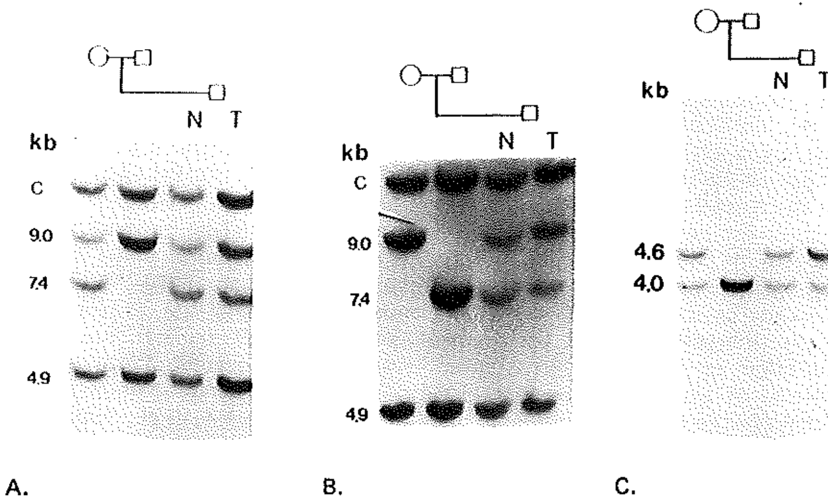


Fig. 4. Southern blot analysis indicating the parental origin of alleles showing imbalances. Tumor specific overrepresentation of 12p is illustrated using the von Willebrand Factor 8 probe. (A) case G002, showing paternal allele overrepresentation (B) case G011, showing maternal allele overrepresentation; chromosome 22 (locus D22S183) (C) case G004, showing overrepresentation of the maternal allele.

Using the von Willebrand Factor 8 probe, tumor specific overrepresentation of the paternal short arm of chromosome 12 in case G002 (A) (more intense signals of the 9.0 kb and the 4.9 kb band (B1 allele) compared to the 7.4 kb band (B2 allele)), and case G011 (B) showing overrepresentation of the maternal 12p in the tumor. In addition, using probe NB84 (locus D22S183), overrepresentation of the maternal chromosomal region 22q11.1-q11.2 of case G004 (C) is illustrated. Four tumors (one SE and three NS) showed overrepresentation of the paternal 12p, while two tumors (one SE and one NS) showed overrepresentation of the maternal 12p. Overrepresentation of the maternal chromosome 22 was found in five tumors (two SE and three NS), while the paternal chromosome 22 was overrepresented in one SE (due to loss of the maternal allele) and in one NS.

Using the Mann-Whitney U test, a significant age difference was found between the patients with a SE or a NS ($p<0.01$), the former being older than the latter (mean 39 versus 29 years of age) (not shown). No age differences were found in the groups of patients with a NS with or without chromosome 12 or 22 allelic imbalances (not shown). In contrast, those patients with a SE showing by molecular techniques overrepresentation of chromosome 22 sequences are significantly younger than those without ($p<0.025$), the former having a similar age as the NS patients. No such difference was found between patients with and without chromosome 22 overrepresentation as detected by karyotyping (mean age of 38 years of both groups).

DISCUSSION

Several loci, localized on the short arms of chromosomes 3, 6, 11 and the long arm of chromosome 12 (Lothe et al., 1989; Radice et al., 1989; Rukstalis et al., 1989; Peltomäki et al., 1990; Murty et al., 1992; Lothe et al., 1993; Looijenga et al., 1993), have been reported to be possibly involved in the pathogenesis of TGCTs by loss of tumor suppressor gene activity, but no consistent pattern has emerged so far. From numerical chromosomal analyses (De Jong et al., 1990; Murty et al., 1990; Rodriquez et al., 1993; Looijenga et al., 1993) it is clear that the process of chromosome loss and gain is not random. Some (parts of) chromosomes are consistently overrepresented, e.g., the short arm of chromosome 12 and the X chromosome (De Jong et al., 1990; Peltomäki et al., 1992; Geurts van Kessel et al., 1993; Rodriquez et al., 1993). Theoretically the involved short arm of chromosome 12 could be of maternal or paternal origin (Geurts van Kessel et al., 1989), or of both (Mukherjee et al., 1991). This question of parental origin of over- and underrepresented (parts of) chromosomes is theoretically relevant in TGCTs because of the effect of the parental origin of certain chromosomal regions in the developmental potential of early embryonic cells (De Groot and Hochberg, 1993 for review). The finding of uniparental origin of the overpresentated short arm of chromosome 12 in isolated TGCTs (Peltomäki et al., 1992; Sinke et al., 1993), and the demonstration that in different cases it may be of either paternal or maternal origin (Peltomäki et al., 1992) is in agreement with our present results (Table 5). Therefore, as expected from the absence of homology of human chromosome 12 sequences with regions of the mouse or rat genome known to be imprinted (O'Brien et al., 1993), and no indication that this chromosome contains imprinted genes from human non-neoplastic and/or neoplastic conditions (De Groot and Hochberg, 1993 for review), this chromosome shows no preferential paternal or maternal involvement in TGCTs.

The marker i(12p) is reported more frequently in NS than in SE (Rodriquez et al., 1992; Rodriquez et al., 1993), which is partly supported by our data showing a higher percentage of 12p overrepresentation in NS compared to SE (Table 2, and Figure 3). The presence of an i(12p) demonstrated by ISH in only 7% of the SE (Rodriquez et al., 1992)

is disconcordant with our molecular data and those of others (Peltomäki et al., 1992), including their own (Samaniego et al., 1990). In contrast to reports on loss of genomic information of region 12q13 and 12q23 in more than 40% of TGCTs (Murty et al., 1990; Murty et al., 1992; Rodriquez et al., 1993), this series, as well as those of others (Radice et al., 1989; Geurts van Kessel et al., 1989; Peltomäki et al., 1990) do not find LOH on 12q in such a high percentage, in spite of using the same probes.

Because of the cytogenetical finding of a relative overrepresentation of chromosome 22 in SE and an underrepresentation in NS (De Jong et al., 1990), we studied the presence of allelic imbalances on this chromosome. Former studies showed no LOH of the chromosomal regions 22q11 and 22q12-q13 in a total of 12 SE, nine NS and four combined tumors (Radice et al., 1989; Peltomäki et al., 1990). Using more cases and more probes, we found aberrations in 58% of TGCTs (62% SE and 54% NS). Uniparental overrepresentation was present in 29% of the tumors (29% of the SE and 29% of the NS), while LOH (partial and complete) was detected in 31% of the tumors (38% of the SE and 25% of the NS). These molecular data appear to be in conflict with the cytogenetic analyses of a large series of TGCTs, showing overrepresentation of chromosome 22 in SE compared to NS (De Jong et al., 1990, and Figure 3). This can be due to the fact that the chromosomal data are derived from direct harvesting of SE, and short term cultures of NS, which might lead to a bias in the results, as recently reported for brain tumors (Rey et al., 1993).

There is a slight overrepresentation of the maternally derived (parts of) chromosome 22, in five cases versus two. In one of the two tumors showing retention of the paternally derived chromosome 22, it was due to LOH of the maternal allele. Thus in five out of six cases where both alleles are present, the maternal allele is overrepresented. A similar kind of disequilibrium is recently reported for acute lymphocytic leukemia, showing deletion of the maternal allele in four out of five cases (Heyman et al., 1993). This finding justifies further investigation into a possible role for genomic imprinting in the development of these tumors.

Patients with a SE showing chromosome 22 overrepresentation using Southern blot analysis were significantly younger than those without ($p<0.025$), and had a similar age as patients with a NS. This suggests a dosage effect of a gene or genes localized on chromosome 22, on the biological behaviour of SE. Possible candidate genes are those encoding the leukemia inhibiting factor (LIF) and oncostatin M (Sutherland et al., 1989; Rose et al., 1993), which are involved in the survival and proliferation or early (primordial) germ cells (Matsui et al., 1992; Resnick et al., 1992).

In conclusion, this study of TGCTs does not pinpoint a region of chromosome 12 or 22 involved in the initiation of TGCTs.

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Chapter VII.

Biallelic Expression of the H19 and IGF2 Genes in Human Testicular Germ Cell Tumors.

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ABSTRACT

Background: Genomic imprinting, resulting in the nonequivalence of expression of homologue genes depending on their parental origin, is an important determinant of the developmental potential of embryonic cells. The expression of two genes, one termed *H19* and the other *IGF2*, has been found to be necessary for proper embryonal development. Both the murine and human *H19* and *IGF2* genes are normally characterized by monoallelic expression. *Purpose:* Because testicular germ cell tumors of adults originate from an early germ cell and, to a certain extent, mimic normal embryonal development, we investigated the patterns of allelic expression of the *H19* and *IGF2* genes in these tumors to determine if genomic imprinting, or a disturbance of it, is involved in their pathogenesis. *Methods:* Specimens of normal tissue and tumor tissue were obtained from 20 patients with testicular germ cell tumors; 10 of the patients had seminomas and 10 had nonseminomatous germ cell tumors. To determine if there was heterozygosity of the *Alu* I and *Apa* I restriction site polymorphism in the *H19* and *IGF2* genes, respectively, DNA was isolated from cells of the peripheral blood of these patients, then subjected to polymerase chain reaction (PCR) amplification, restriction enzyme digestion, and electrophoresis in agarose gels. If heterozygosity was determined, a similar analysis was performed on complementary DNA (cDNA) obtained from matched tumor RNA by reverse transcription and subsequent PCR amplification (RT-PCR). If monoallelic expression was found, matched tumor DNA was studied for possible deletions. *Results:* Tumor samples from 14 of 20 and 11 of 20 patients were informative for allelic expression patterns of the *H19* and *IGF2* genes, respectively. Analysis of the products of the RT-PCR showed biallelic expression of the *H19* gene in 12 testicular germ cell tumors (patients numbered 6, 8-13, 15, 16, and 18-20) and of the *IGF2* gene in 10 testicular germ cell tumors (patients numbered 1, 3, 6, 8-13, and 15-20). The three remaining tumors (patients numbered 2, 4, and 5) had lost the nonexpressed allele. *Conclusions:* In contrast to normally developing embryos, testicular germ cell tumors show a consistent expression of both parental alleles of the *H19* and *IGF2* genes. *Implications:* Testicular germ cell tumors of adults may either develop from precursor cells in which the imprinting has been either erased or subjected to a consistent relaxation of its effect.

INTRODUCTION

The factors involved in the initiation, promotion and progression of testicular germ cell tumors of adults are largely unknown (1,2). These tumors originate from a common precursor (3), referred to as carcinoma in situ. The initiation of tumorigenesis probably occurs in utero (4,5), during the maturation of primordial germ cells into gonocytes (2).

Histologically, testicular germ cell tumors can be composed of embryonal and/or extraembryonal tissues (6). The balance between these two differentiation lineages during murine and human development is affected by the parental origin of certain chromosomal regions (7-13). Four mouse genes (*H19, Igf2, Igf2*-receptor, and *Snrpn*) have been reported to show differential expression depending on their parental origin (defined as genomic imprinting) (14-17). The first two genes are imprinted similarly in humans and in mice, showing expression of only the maternally derived allele of the *H19* gene and of only the paternally derived allele of the *IGF2* gene (14,15,18-23). Both genes are necessary for proper embryonal development (19,22).

Because fertilization results from fusion of a haploid set of chromosomes derived from the father (sperm) and the mother (oocyte), the genome of the zygote contains both paternally and maternally imprinted chromosomes. Subsequently, during gametogenesis, the imprinting pattern of genes in differentiating germ cells is changed to either a completely maternal or paternal pattern (24). Little is known about the molecular basis and timing of this process. In view of the cell of origin, and the developmental potential of testicular germ cell

tumors, it is conceivable that genomic imprinting (or a disturbance of it) has a role in their pathogenesis. The finding of preferential loss of the paternal short arm of chromosome 11 (25) suggests that genomic imprinting is indeed involved in the pathogenesis of these tumors. We studied the (preserved) monoalleleic and (acquired) biallelic expression of the *H19* and *IGF2* genes in primary testicular germ cell tumors.

MATERIALS AND METHODS

Control and tumor tissue specimens were obtained from 20 patients who had been diagnosed as having primary testicular germ cell tumors. Using the World Health Organization classification (6), 10 patients had seminomas and 10 had nonseminomatous tumors. In summary, the study design was as follows: all DNA samples isolated from peripheral blood were studied for heterozygosity of the *H19* and *IGF2* genes. The tumors of the individuals heterozygous for one or both gene loci were analyzed for monoallelic or biallelic expression of the two genes. In addition, the tumors showing monoallelic expression were studied for possible loss of the nonexpressed allele.

High molecular weight genomic DNA from peripheral blood of the patients was isolated by proteinase K-sodium dodecyl sulfate treatment followed by phenol-chloroform extraction and ethanol precipitation (26). Total RNA was prepared from the tumor cells by the guanidinium-cesium chloride method (27). RNA (2 μg) was then treated with RNase free DNase (type I; Promega Biotec, Madison, Wis.) for at least 20 minutes at 37 °C at a final concentration of 5 U per 20 μL and ethanol precipitated. The pellet was resuspended in 20 μL of 1X transcription buffer (Gibco BRL, Breda, The Netherlands) containing 1 mM deoxynucleoside triphosphates (Pharmacia P-L Biochemicals INC, Milwaukee, Wis.), 100 ng random primer Pd(N)6, and 4.5 U of RNasin (Pharmacia P-L Biochemicals Inc.). The pellet was then divided in half, and 200 U Moloney murine leukemia virus reverse transcriptase (Gibco BRL) was added to one sample. The other half was used as negative control to rule out genomic DNA contamination during amplification of the complementary DNA (cDNA) by PCR. Reverse transcription was performed at 37 °C for 1 hour.

All amplifications were performed in 1X standard buffer (A) as described by the supplier (Promega Biotec).

The primer sequences and concentrations for amplification of both genomic DNAs and cDNAs of the *H19* gene have been reported previously (20), i.e., primer 1 (AACACCTTAGGCTGGTGG), primer 2 (TGCTGAAGCCCTGGTGGG), primer 3 (CACTATGGCTGCCCTCTG), and primer 4 (TCGGAGCTTCCAGACTAG). After an initial denaturation of 4 minutes at 94 °C, every amplification cycle consisted of denaturation for 1.5 minutes at 94 °C, annealing for 1 minute at 56 °C, and extension for 1.5 minutes at 72 °C. For amplification, 1 μg of DNA or cDNA was subjected to 20 amplification cycles, using primers 1 and 2 in a total volume of 50 μL buffer A. Subsequently, 1 μL was diluted in 49 μL buffer A containing fresh reagents and subjected to an additional 30 cycles, using primers 3 and 4.

To verify proper amplification (indicated by the absence of products in the negative control) as well as the sizes of the fragments (i.e., 828 bp [bp] for DNA and 668 bp for cDNA) after the second amplification 5 μL of the solution was loaded on a 1.5% agarose gel and interpreted after electrophoresis and ethidium bromide staining. The procedure was continued only when the negative control lacked amplification products.

The amplification products were ethanol precipitated and digested to completion with 8 U of *Alu* I endonuclease according to the manufacturer's instructions (Pharmacia P-L Biochemicals Inc.). The products were then separated on a 1.5% agarose ethidium bromide gel. Those DNA samples showing an uncleaved 298-bp, a constant 261-bp band, and a 151/147-bp band were considered heterozygous (20). The matched tumor RNAs were judged

as biallelic when the cDNA exhibited the constant 261-bp band and both the uncleaved 219-bp and 147-bp bands were present. The 72-bp fragment (219 - 147 bp) was not apparant using these electrophoretic separation conditions. The size difference between the uncleaved DNA fragment and the uncleaved cDNA fragment (i.e., 298 and 219 bp) is due to the presence of the intron DNA sequence between exon 4 and 5 in the former (20).

Primer sequences for amplification of genomic DNAs and cDNAs for the *IGF2* gene have been described (28): primer 1 (forward primer) (CTTGGACTTTGAGTCAAATTGG), primer 2 (reverse primer) (CCTCCTTTGGTCTTACTGGG). The same controls for proper amplification as described for the *H19* gene analysis were included. After initial denaturation for 5 minutes at 94 °C, a subsequent denaturation for 1 minute at 95 °C, annealing for 2 minutes at 56 °C, and extension for 1 minute at 72 °C for 40 cycles were performed. For cDNA amplification, the following conditions were applied: 5 minutes initial denaturation at 94 °C, subsequent denaturation for 1 minute at 95 °C, and annealing and extension at 56 °C for 2 minutes for 40 cycles. Amplification products were ethanol precipitated and subsequently digested with 10 U of *Apa* I endonuclease. Digestion products were separated on a 1.5% agarose ethidium bromide gel. As introns are not present in the region of the *IGF2* gene that was amplified, the uncleaved amplification products derived from DNA of the gene and the cDNA are of the same size (i.e., 236 bp). Those cases showing both 236- and 173-bp DNA fragments after *Apa* I digestion are heterozygous. The 63-bp fragment (236 - 173 bp) was not apparant with the separation conditions used. The presence of both the 236- and 173-bp fragments in the cDNA samples indicated biallelic expression (28).

RESULTS

Both seminomas and nonseminomatous TGCTs, the latter with different histologies, were included in this study (Table 1).

The results of the screening for informativity (meaning heterozygosity of the alleles of the *H19* and *IGF2* genes) as well as expression analysis for the heterozygous cases are summarized in Table 1 and illustrated in the Figs. 1 and 2. The study was restricted to DNA from normal tissue (peripheral blood cells) when the analysis of that DNA from the patient exhibited noninformativity (upper part of both figures). RNA analysis was included when the patient was informative (middle part of both figures). All controls for possible DNA contamination in the RNA samples lacked amplification products. Tumor DNA was studied when the patient was informative and when monoallelic expression was found in the tumor (lower parts of Figs. 1 and 2).

Six patients were homozygous for *H19* (patients 2, 3, 4, 5, 7, and 17) (Fig. 1). Twelve testicular germ cell tumors (six seminomas and six nonseminomatous germ cell tumors) from 14 individuals heterozygous for *H19* showed biallelic expression of this gene (patients 6, 8, 9, 10, 11, 12, 13, 15, 16, 18, 19, and 20). Two seminomas (patients 1 and 14) showed heterozygosity using control DNA from normal tissue and monoallelic expression of *H19* in the tumor. Both tumors contained only the expressed allele, while the other allele was lost. A faint band of 298 bp was visible in the DNA analysis of tumor 1, most probably caused by DNA contamination of normal host tissue (*see* the "Discussion" section).

Nine patients were homozygous for *IGF2* (patients 2, 4, 5, 7, 8, 14, 15, 18, and 19) (Fig. 2). Ten patients (four seminomas and six nonseminomatous germ cell tumors) showed heterozygosity, using control DNA and biallelic expression of the *IGF2* gene in the tumor (patients 1, 3, 6, 10, 11, 12, 13, 16, 17, and 20). One seminoma (patient 9) showed the presence of both alleles in control DNA but monoallelic expression in the tumor; this was determined to result from loss of the nonexpressed allele.

Table 1. Summary of the histology of the studied tumors and the results of the analysis of biallelic or monoallelic expression of the *H19* and *IGF2* genes

Patient No.	Histology'	*H19*: *Alu* I⁺		*IGF2*: *Apa* I⁺	
		Informativity	Expression	Informativity	Expression
1	SE	A/B	B	A/B	A/B
2	NS,YS,TE(M)	B		A	
3	NS;EC	B		A/B	A/B
4	NS;EC,YS,TE(M+I)	B		B	
5	SE	A		A	
6	NS;EC	A/B	A/B	A/B	A/B
7	NS;EC,YS,TE(M+I)	B		B	
8	NS;TE(M+I)	A/B	A/B	B	
9	SE	A/B	A/B	A/B	A
10	SE	A/B	A/B	A/B	A/B
11	SE	A/B	A/B	A/B	A/B
12	SE	A/B	A/B	A/B	A/B
13	NS;EC,YS,TE(M+I)	A/B	A/B	A/B	A/B
14	SE	A/B	B	A	
15	SE	A/B	A/B	A	
16	NS;TE(M+I)	A/B	A/B	A/B	A/B
17	NS;CH,TE(M)	A		A/B	A/B
18	SE	A/B	A/B	B	
19	SE	A/B	A/B	A	
20	NS;EC,TE(I)	A/B	A/B	A/B	A/B

' SE = seminoma; NS = nonseminomatous germ cell tumor; EC = embryonal carcinoma; YS = yolk sac tumor; CH = choriocarcinoma; TE = teratoma (M = mature; I = immature). ⁺ A = A allele; B = B allele.

Seven testicular germ cell tumors, three seminomas and four nonseminomatous germ cell tumors, showed biallelic expression of both *H19* and *IGF2* genes (patients 6, 10, 11, 12, 13, 16, and 20).

In summary, both parental alleles of the *H19* and *IGF2* genes were expressed in most testicular germ cell tumors.

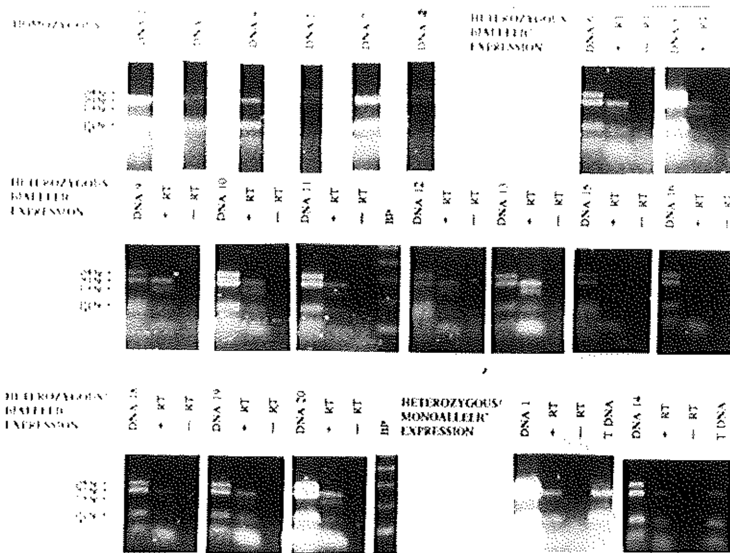

Fig. 1. Alu-I restriction patterns of polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR products of the H19 gene of 20 testicular germ cell tumors. Arrows indicate restriction sizes for DNA (298 bp, 261 bp, and 151/147 bp) and cDNA ([lane indicated by +RT] 261 bp, 219 bp and 147 bp), T DNA = tumor DNA. All negative controls (— RT) as well as a 100-bp ladder (BP) are shown.

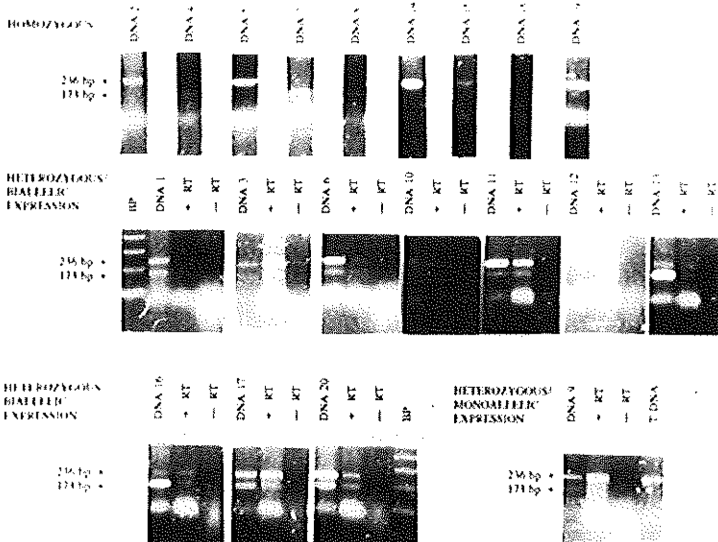

Fig. 2. Apa-I restriction patterns of polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR products of the IGF2 gene of 20 testicular germ cell tumors. Arrows indicate restriction sizes for DNA and cDNA ([lane indicated by + RT]) 236 bp and 173 bp), T DNA = tumor DNA. All negative controls (— RT) as well as a 100-bp ladder (BP) are shown.

DISCUSSION

During the last decade, a new regulatory mechanism crucial for the proper development of both embryonal and extraembryonal tissues has been identified (7). It is characterized by a nonequivalence of expression of homologue genes depending on their parental origin - referred to as genomic imprinting. Experiments in mice and rats that have involved the inducing of a partial or complete uniparental composition of the genome, as well as spontaneous human pathological conditions, illustrate the importance of this mechanism for normal development (7-10,12,13,15,29-33). The human and mouse *H19* and *IGF2* genes are imprinted similarly (18,20,22,23). Disturbance of the physiological control of the expression of the *H19* and *IGF2* genes results in malformation or lethality during early murine development (15,34,35). In addition, loss of the maternal and duplication of the paternal chromosomal band 11p15.5, containing both the *H19* and *IGF2* genes (36), during human development has been associated with the Beckwith-Wiedemann Syndrome, which is characterized by a severe growth disturbance and predisposition for development of embryonal tumors (37). Histologically similar embryonal tumors that are not related to this syndrome also show specific deletion of maternal chromosomal region 11p15.5 (38-40). In contrast, testicular germ cell tumors show a preferential loss of the paternally derived region and retention of the maternally derived region (25). These data suggest a role for genomic imprinting in the pathogenesis of these tumors, but the reason for the opposite pattern of loss and retention of the parental regions is unknown.

In the context of genomic imprinting, testicular germ cell tumors are of special interest. They may be composed of embryonal and/or extraembryonal tissues, showing analogy with the outcome of murine pronuclear transfer experiments (7). Moreover, these tumors probably originate from stem cells, developmentally positioned between primordial germ cells and gonocytes (3), somewhere along the route where paternal or maternal imprinting (depending on the sexe of the individual) is erased and new imprinting takes place (24). Expression patterns of these genes in testicular germ cell tumors reveal these processes. Therefore, we investigated whether these tumors show a monoallelic or biallelic expression of the *H19* and *IGF2* genes. From a series of 20 cases (10 seminomas and 10 nonseminomatous germ cell tumors) 16 cases could be studied for their monoallelic or biallelic expression of either one or both of these genes. In contrast to the situation in normal development, testicular germ cell tumors consistently show expression of both parental alleles. Biallelic expression of both the *H19* and *IGF2* was found in seven tumors, irrespective of histology. Monoallelic expression was only found when one of the alleles had been lost. Patient 14 showed monoallelic expression of the *H19* gene, but no data were available for *IGF2* because of homozygosity. Biallelic expression of the *H19* gene was present in patient 9, but only the allele without the *Apa* I restriction site of the *IGF2* gene was detected. In contrast, in patient 1, biallelic expression was found for *IGF2*, but *H19* showed only expression of the allele with the *Alu* I restriction site. The faint 298-bp band in the corresponding DNA sample may have been caused by contamination with DNA from non-malignant host tissue, most probably lymphocytes, which can be found abundantly in seminomas (41). Histological examination supported this interpretation. Using RNase protection analysis, we are currently investigating whether the observed difference in the intensities of the levels of expression of the parental alleles that are detected by RT-PCR and subsequent endonuclease digestion (primarily present for *IGF2*) reflects the in vivo situation.

Despite the close proximity of the *H19* and *IGF2* genes on chromosomal band 11p15.5 (36), two patients (patients 1 and 9) showed deletion of only one of these genes and biallelic expression of the other. This finding is interesting because of the reported relatively frequent loss of chromosomal band 11p15.5 in testicular germ cell tumors (25,42-44). Because of the incidence of deletions affecting the short arm of chromosome 11, we proposed that loss of 11p sequences is probably a late or noncrucial event in the

pathogenesis of these tumors (44). The possible relationship between biallelic expression of the imprinted genes *H19* and *IGF2* and occurrence of deletions affecting these genes is currently under investigation.

The biallelic expression of both the *H19* and *IGF2* genes in testicular germ cell tumors may be caused by an erased imprint or a paternally imprinting status of the tumor cells (45). However, the developmental potential of these tumors, showing both embryonal and extraembryonal tissues (6), is not in agreement with a complete paternal imprinting pattern (46). In addition, a completely erased status is unlikely because of the preferential loss of the short arm of paternal chromosome 11 (25), which demands retention of nonequivalence of the paternally and maternally derived chromosomes. Retention of the paternal imprinting and conversion of the maternal imprinting during passage through the male germ line, and visa versa during passage through the female germ line (24), could explain the specific involvement of one of the parental chromosomes 11, as well as the biallelic expression of the imprinted genes *H19* and *IGF2*.

Loss of imprinting of the *IGF2* and *H19* genes might be the alternative. This loss was recently reported in a series of renal Wilms' tumors and a rhabdoid tumor (47,48). In total, two of seven tumors showed biallelic expression of the *H19* gene (48), and 14 of 17 for *IGF2* (47,48). Two of the tumors with monoallelic expression of the *IGF2* gene showed loss of the nonexpressed allele (47). Both genes showed loss of imprinting in one out of three tumors (48). The consistency of biallelic expression of the imprinted genes in testicular germ cell tumors observed in this study in intriguing. Also of interest is the recently observed disturbance in the methylation pattern of the *H19* gene during early development (21). This alteration of methylation can result not only in an allelic shift of expression, but, theoretically , also in a total lack of silencing in a subpopulation of early stem cells (49), which might be the case for the precursor of these tumors. In this context, it is interesting to note that constitutive biallelic expression of the *IGF2* gene has been observed in a patient with Beckwith-Wiedemann syndrome (50) and in fibroblast cultures of patients with this disease (51) as well.

We propose that testicular germ cell tumors originate from precursor cells that still contain nonequivalent paternal and maternal haploid sets of chromosomes, possibly due to a pre-erased or post-erased imprinting status. The former is in keeping with the potential of the cell of origin to differentiate into both somatic and trophoblastic lineages, which requires maternal and paternal imprinting, respectively. The specific chromosomal constitution of testicular germ cell tumors (52,53), acquired during tumor progression, probably affects the developmental potential and, as a corollary the histological constitution and the biological behavior of these tumors.

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Chapter VIII.

CHROMOSOME 15 ALLELIC IMBALANCES AND PARENTAL METHYLATION STATUS IN GERM CELL TUMORS OF THE ADULT TESTIS.

(Submitted).

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ABSTRACT

Chromosomal analyses show a consistent overrepresentation of chromosome 15 in seminomas (SE) compared to nonseminomatous testicular germ cell tumors of adults (NS). Therefore, genes localized on this chromosome may be involved in controlling differentiation to either direction from their common precursor. Southern blot analysis in a series of 20 SE and 25 NS supported the numerical difference between chromosome 15 in SE and NS; overrepresentation was only found in SE (25%), while loss was present in both SE and NS, accounting for in total less than 20%.

The parent dependent methylation status of chromosomal region 15q11-q13, detected by the cDNA probe DN34 after digestion with the methylation-sensitive endonucleases *HpaII* and *HhaI*, was studied in a series of these SE and NS. Similar to DNA isolated from peripheral blood (n=5) and testis parenchyma without active spermatogenesis (n=5), all NS (n=12) as well as two out of 17 SE (12%) showed a biparental methylation pattern. Eight SE showed a pattern of demethylation which could be explained by allelic imbalances in the tumor. Five SE without allelic imbalances of chromosome 15 showed a paternal-like methylation pattern comparable to DNA of testis parenchyma with spermatogenesis (n=6). In spite of showing mainly this paternal methylation pattern, one SE showed a relative overrepresentation of the maternal allele. We conclude that these tumors originate from a pre-erased precursor, and that SE show different methylation patterns, possibly reflecting differentiation into the germ lineage.

INTRODUCTION

All testicular germ cell tumors of adults (TGCTs) originate from carcinoma in situ (CIS) (1), which most probably starts to develop already in utero (2-4). CIS shows morphological and immunohistochemical characteristics of early (primordial) germ cells (1,5). The invasive tumors can be composed of cells being highly similar to CIS cells, the seminoma (SE), and/or elements containing embryonal and/or extraembryonal cell lineages, the nonseminomatous TGCTs (NS) (6). The factors controlling differentiation to either of these or both lineages are largely unknown (7,8).

Murine and human non-neoplastic and neoplastic pathological conditions illustrate that the parental origin of certain parts of the genome are regulatory in the developmental potential of early embryonal cells (9-12). This is due to non-equivalence of certain genes depending on their parental origin, referred to as genomic imprinting (13). Because genomic imprinting is sex dependent, a transition from a biparental pattern to either a maternal or paternal pattern has to occur during maturation of the primordial germ cell to the mature gamete (14). This is somewhere in the route where the initiation of the pathogenesis of TGCTs is suggested to occur (5).

In general, methylation is known to be involved in regulation of gene expression (15,16). Recently methylation is also suggested to be the mechanism for silencing certain genes subjected to genomic imprinting (17,18). Indeed a change in the methylation status during maturation from the primordial germ cells to the mature gamete has been reported (19,20). In this context, the overall hypermethylation of NS and demethylation of SE is interesting (21). Recently a parent dependent methylation polymorphism on chromosome 15, band q11-q13 has been described (22). This is used as a tool to study the uni- or biparental methylation pattern of a series of 19 SE and 12 NS, as well as in testes with and without spermatogenesis, indicative for the status of imprinting. This approach may give indications on which cell is the normal counterpart of the CIS cell, the timing of initiation, as well as about factors controlling the development of TGCTs. To correct for possible allelic imbalances, the tumors were studied by Southern blot analysis. In addition,

this series was extended to 20 SE and 25 NS to investigate the overall involvement of allelic imbalances of chromosome 15 in the pathogenesis of TGCTs.

MATERIAL AND METHODS

Samples

Forty-five fresh primary TGCTs were collected in the Netherlands. They were classified according to the WHO-classification (6) using representative frozen and paraffin embedded tissue sections, stained with hematoxylin and eosin. If necessary, the diagnosis was supported using immunohistochemistry as described (23). Twenty tumors were diagnosed as SE, and 25 as NS (see Table 1). Testis parenchyma of 11 patients (four with a SE and seven with a NS) were available. Parenchyma adjacent to tumor tissue was screened for the presence of spermatogenesis using conventional light microscopy of hematoxylin and eosin stained tissue sections. After informed consent, heparinized peripheral blood was collected from those patients of whom testis parenchyma was not available.

Southern Blot Analysis

High molecular-weight DNA was extracted from each sample using standard procedures (sodium dodecyl sulfate-proteinase K and phenol-chloroform extraction and ethanol precipitation) (24). Light microscopically all tumor samples were ascertained to contain at least 60% tumor cells. Ten micrograms of DNA was digested to completion with the appropriate restriction endonucleases according to the manufacturer's prescription (Pharmacia, Woerden, NL). Fragments were electrophoresed on a 0.8% agarose gel with a Tris-acetate buffer, and transferred to nylon membrane (Hybond N+; Amersham, Inc, Arlington Heights, Ill) according to Southern (25). DNA loading variation was checked by visual judgement of ethidium bromide stained gels. In addition, probe IGH (26) was used as reference. This probe did not show a high frequency of allelic imbalances in a large series of TGCTs (unpublished results). After hybridization the filters were washed until two times 0.1xSSC/0.1% SDS, for 15 minutes at 65°C and exposed to HPX-44 Medical X-Ray film (Valca, Spain) for 1-5 days at -70°C.

Screening for Allelic Imbalances

To study the presence of allelic imbalances of chromosome 15, the tumor samples and matched controls (DNA isolated from peripheral blood or testis parenchyma) were studied with the following polymorphic probes, loci are indicated between brackets; pCMW-1 [D15S24], mapped to 15pter-q13, pD151 [D15S2], mapped to 15q15-q22, as well as the non-polymorphic probes *IGF*I-R and hu-c-*FES*, both mapped to 15q25-qter. Information about the probes can be found in the HGM 11 report (26). All probes were radioactively (^{32}P-dATP; Amersham) labeled using random primer synthesis (Boehringer, Mannheim). Filters were rehybridized with a different DNA probe after the previous hybridization signals had been stripped off. Allelic imbalances were analyzed by densitometric screening (Biorad) and interpreted as described (27).

Table 1.
Age of the patient at diagnosis and histology of the 45 primary germ cell tumors of the adult testis.
The tumors studied by the cDNA probe DN34 are indicated (+).

Case	Age (yrs)	Histology	Components	DN34
001	22	NS	EC;CH;TE(M+I)	+
02	39	SE		+
03	20	NS	EC	
04	29	SE		+
06	24	NS	EC	+
07	46	NS	TE(M);EC	
08	33	NS	EC;TE(M);CH;YS	
09	45	SE		+
10	39	SE		+
11	37	SE		+
12	47	SE		+
13	29	SE		+
14	31	SE		+
15	19	NS	EC;YS	+
16	23	NS	EC;TE(M+I);YS	+
17	43	NS	TE(M);CH	+
18	16	NS	EC	
19	51	NS	EC;YS	
20	29	NS	EC	
21	22	NS	EC;TE(M+I);CH	
22	21	NS	EC;TE(M+I);YS	+
23	71	SE		+
24	30	SE		+
25	30	SE		
26	64	NS	EC	
28	28	SE		+
29	29	SE		+
30	46	SE		+
31	29	NS	TE;EC	
32	26	NS	TE;EC	
33	59	SE		+
34	37	NS	EC;TE;YS	+
35	20	NS	TE(M+I)	
36	36	SE		+
37	23	NS	EC;TE(M);CH	+
38	53	SE		+
39	39	SE		+
41	21	NS	EC;TE(M);YS	
42	31	NS	EC;TE(M)	+
43	22	NS	EC;TE(M+I);YS	
44	30	NS	EC	+
45	28	NS	EC;TE(M+I);YS	+
46	23	NS	TE(M+I);YS	+
47	25	SE		+
48	32	SE		+

Abbr. SE = seminoma; NS = nonseminomatous germ cell tumor; EC = embryonal carcinoma; CH = choriocarcinoma; TE = teratoma (M = mature; I = immature); YS = yolk sac tumor.

Methylation Analysis

The methylation status of chromosomal region 15q11-q13, as detected by the complete cDNA probe DN34 (22), is studied in a subseries of samples, comprising five peripheral blood samples (of cases G001, 006, 010, 011, 030) five testes without spermatogenesis (of cases G015, 016, 022, 028, 036), six testes with spermatogenesis (of cases G012, 017, 018, 020, 024, 035), 12 NS and 19 SE (indicated in Table 1). Briefly, 20 microgram high molecular weight DNA was digested to completion with the restriction endonuclease *Eco*RI. After precipitation, it was split into two identical fractions. One part was digested with *Msp*I, and the other with the methylation-sensitive restriction endonuclease *Hpa*II. Another 10 microgram DNA was double-digested with *Hin*dIII and the methylation-sensitive restriction endonuclease *Hha*I. Electrophoresis, Southern blotting, and hybridization was performed as described above. Autoradiograms were analyzed with a densitometric screening (Biorad, Richmond, CA), subtracting the background signal. Peak height and area under the curve indicate band intensity.

RESULTS

The results of the analysis for allelic imbalances on chromosome 15 in a series of 20 SE and 25 NS are shown in Figure 1 and summarized in Table 2.

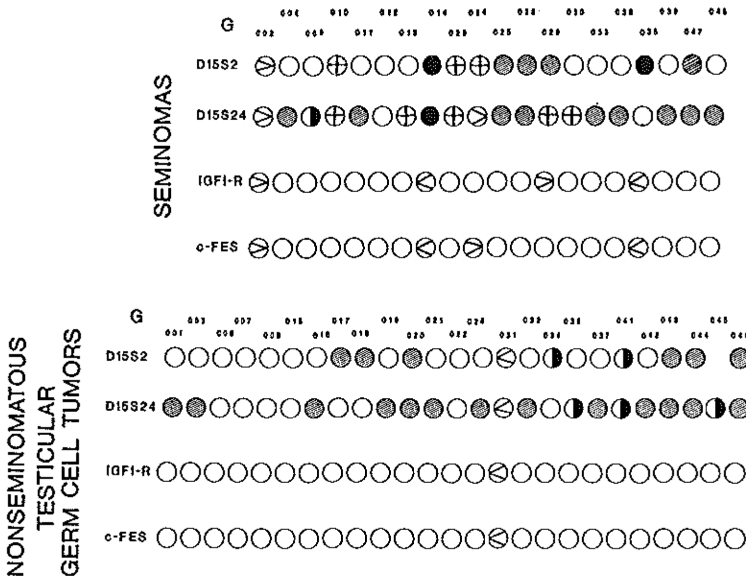

Fig. 1. Overview of the results of Southern blot analysis of chromosome 15 in 20 seminomas and 25 nonseminomatous testicular germ cell tumors, using two informative and two non-informative probes (○ = non-informative; ⊖ = non-informative and overrepresentation; ⊖ = non-informative and underrepresentation; ◍ = informative without allelic imbalances; ⊕ = informative and overrepresentation; ◑ = partial loss of heterozygosity; ● = complete heterozygosity).

Table 2.
Overview of the percentages of tumors affected (only of the informative cases for D15S2 and D15S24) on chromosome 15, in total and separately for the seminomas (SE) and nonseminomatous testicular germ cell tumors (NS).

Locus	Informative		Overrepresentation			Loss of Heterozygosity					
						Complete			Partial		
	SE	NS	SE	NS	T	SE	NS	T	SE	NS	T
D15S2	9/20	8/24	33	0	18	22	0	12	0	25	12
D15S24	16/20	16/25	31	0	16	6	0	3	6	19	13
								SE	NS		
IGF1-R	-	-	10	0	4			10	4		
hu-c-*FES*	-	-	10	0	4			10	4		

None of the NS contained overrepresentation of chromosome 15 sequences, while it was present in 33% of the SE. In contrast, both SE and NS showed deletions of (parts of) chromosome 15, i.e., complete loss of heterozygosity only in SE, and partial loss of heterozygosity mainly in NS. Most frequently all loci along the chromosome were involved, suggesting that the whole chromosome is involved.

Using the cDNA probe DN34, locus D15S9, the parentally determined methylation status was studied in a series of DNA samples isolated from peripheral blood, testis parenchyma with and without spermatogenesis, as well as SE and NS. The DNA fragments detected after digestion with the different restriction endonucleases and subsequent Southern blot analysis, are summarized in Table 3. All DNAs isolated from peripheral blood, testis parenchyma without spermatogenesis and NS reveal a biparental methylation pattern using *Hpa*II (the 5.2, 4.3, 4.0, and 3.5 kb restriction fragments were present in comparable intensities). This was supported by the results obtained from the *Hha*I digestions, showing the 4.5, 3.5, 3.1, 2.9, 2.6, 2.0, 1.6, 1.5, and 1.0 kb bands, of which the 4.5 and 1.0 kb bands are most intense. This is illustrated in Figure 2A. In addition, the similar pattern of DNA isolated from testis parenchyma without spermatogenesis (N-) and from a NS (G022), both digested with *Hha*I, is illustrated. In contrast, DNA from testis parenchyma with spermatogenesis show mainly the 3.5 kb band after digestion with *Hpa*II, and the 3.5 and 2.9 kb bands using *Hha*I, illustrated in Figure 2B.

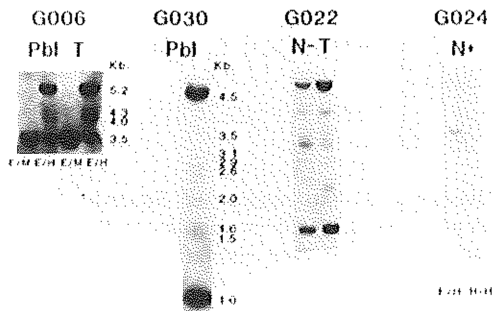

Fig. 2. Representative examples of the methylation status of chromosomal region 15q11-q13 detected by the cDNA probe DN34 after digestion with EcoRI/MspI (E/M), EcoRI/HpaII (E/H), or HindIII/Hha I (H/H) as indicated. Illustrated are DNA samples of (A) case G006: peripheral blood (Pbl) and tumor (T) digested with E/M and E/H; G030: Pbl H/H; G022: testis parenchyma without spermatogenesis (N-) and T both with H/H; (B) case G024: testis parenchyma with spermatogenesis (N+) E/H H/H.

Table 3
Summary of the methylation analysis of DNA digested with either *Hpa*II or *Hha*I and subsequent hybridization with the cDNA probe DN34 of peripheral blood (Pbl), testis parenchyma without spermatogenesis (N-), testis parenchyma with spermatogenesis (N+), as well as seminomas (SE) and nonseminomatous germ cell tumors (NS). The sizes (kb) of the DNA fragments after hybridization and their relative intensities (5 = + + + + +; 4 = + + + +; 3 = + + +; 2 = + +; 1 = +/-; 0 = -; ND = not determined) are indicated.

*Eco*RI/*Hpa*II				Endonuclease	*Hin*dIII/*Hha*I								
5.2	4.3	4.0	3.5	fragment size (kb)	4.5	3.5	3.1	2.9	2.6	2.0	1.6	1.5	1.0
				Sample									
5	4	4	5	Pbl, N-, NS	5	3	3	3	3	2	3	3	5
2	0	0	5	N+	1	3	0	2	0	0	1	1	1
1	0	0	4	002	5	3	0	4	0	0	2	0	1
1	0	0	4	004	4	1	1	4	1	1	4	1	2
1	0	0	4	009	5	3	3	3	3	3	2	2	3
2	1	1	4	010	5	3	1	4	0	0	2	2	4
1	0	0	4	011	4	4	0	1	0	0	4	1	2
4	4	4	4	012	ND								
1	0	0	4	013	4	4	0	4	1	1	0	1	3
1	0	0	5	014	4	4	2	5	1	1	0	1	2
1	0	0	4	023	4	3	3	3	2	1	2	2	3
1	0	0	4	024	5	3	2	2	3	1	2	2	2
1	0	0	4	028	2	4	2	4	2	2	2	1	3
1	2	2	5	029	4	1	1	1	3	4	0	2	3
3	4	4	4	030	ND								
5	4	4	5	033	5	4	3	3	4	2	3	2	4
1	0	0	4	036	4	3	2	5	2	1	1	1	5
1	2	2	5	038	5	2	3	2	2	3	2	2	5
5	4	4	5	039	5	4	3	3	4	2	3	2	4
5	0	0	5	047	4	4	2	4	4	2	5	2	3
4	4	4	4	048	4	4	2	4	0	2	2	2	4

Nineteen SE were studied after *Hpa*II, and 17 also after *Hha*I digestion (excluding cases G012 and G030, both showing a biparental methylation pattern). A biparental methylation pattern using both restriction endonucleases was found in two SE (cases G033 and G039), illustrated in Figure 3A. These tumors showed no allelic imbalances (see Figure 1). Four tumors (cases G009, 023, G024, and G048) showed disconcordance between the *Hpa*II and *Hha*I results. The first three tumors showed demethylation using *Hpa*II (revealing mainly the 3.5 kb band), while a mainly biparental pattern was found using *Hha*I, illustrated in Figure 3B for case G024. These tumors contained allelic imbalances, i.e., partial loss of the maternal allele in case G009 (not shown) and overrepresentation of

an allele of unknown origin in cases G023 and 024 (see Figure 1). The other tumor (G048) showed no allelic imbalances, a biparental pattern using *Hpa*II, but a mainly demethylated (paternal) pattern was detected using *Hha*I. Case G014 showed complete tumor specific loss of heterozygosity (loci D15S2 and D15S24, Figure 1 and 3C), and revealed a paternal-like methylation pattern using both methylation-sensitive restriction enzymes (Table 3 and Figure 3C). This could be explained assuming loss of the maternal allele, which was not possible to verify because of the lack of parental DNA. A partial demethylation pattern was found in two other tumors (cases G029 and G038), illustrated in Figure 3D, which could be due to the allelic imbalances present, assuming overrepresentation of the paternal and loss of the maternal allele, respectively (see Figure 1). Again, this could not be verified. Eight SE (cases G002, 004, 010, 011, 013, 028, 036, 047) showed a methylation pattern comparable to the pattern found in DNA of testis parenchyma with spermatogenesis, illustrated in Figure 3E for G002 and 036. Two out of these SE contained allelic imbalances of chromosome 15 sequences in the tumor (G002 and 013), which might be the molecular basis for the methylation pattern found, assuming a relative overrepresentation of the paternal allele. While a relative overrepresentation of the maternal compared to the paternal allele (locus D15S2 and D15S24) was found for case G010 (Figure 1 and 4), a mainly paternal-like methylation pattern was identified after *Hpa*II and *Hha*I digestion (Table 3 and Figure 4).

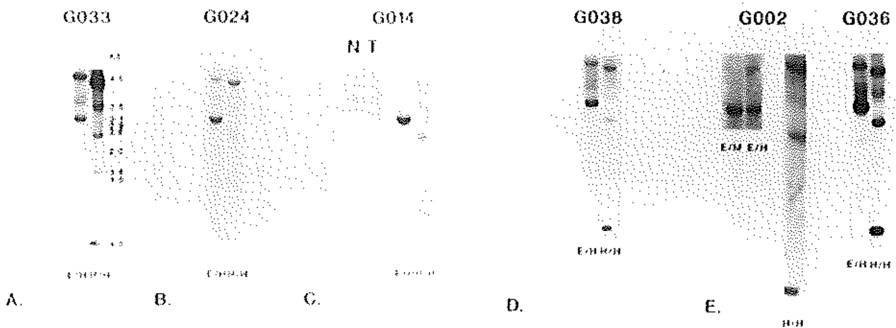

Fig. 3. Examples of the different patterns of methylation as detected in seminomas by the cDNA probe DN34 after digestion of DNA with EcoRI/MspI (E/M), EcoRI/HpaII (E/H), or HindIII/HhaI (H/H) as indicated. Illustrated are (A) case G033 E/H H/H, showing a biparental methylation status for both digestions; (B) case G024 E/H showing a demethylation, and H/H showing a biparental methylation; (C) case G014 showing tumor specific loss of heterozygosity on locus D15S2, and a paternal methylation pattern using E/H and H/H; (D) case G038 showing partial demethylation using E/H and H/H; (E) case G002 showing a paternal methylation pattern using E/H and H/H, and case G036 also showing a paternal methylation pattern using E/H and H/H.

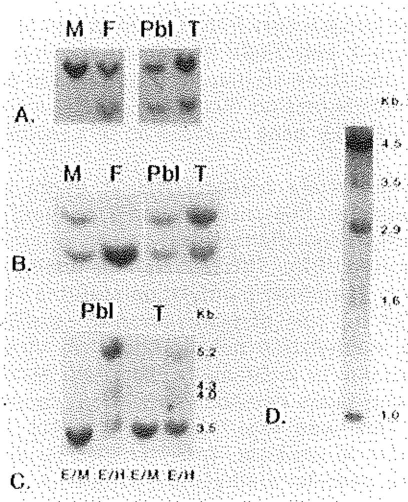

Fig. 4.
Results of case G010, showing tumor (T) specific overrepresentation of the maternal allele (M=mother, F=father) on locus (A) D15S2 and (B) D15S24, after digestion with EcoRI. Methylation status detected by the cDNA probe DN34 after digestion with EcoRI/MspI (E/M), EcoRI/HpaII (E/H), or HindIII/HhaI (H/H), showing (C) peripheral blood (Pbl) and T DNA digested with E/M and E/H; (D) T with H/H.

DISCUSSION

Pronuclear transfer experiments as well as analysis of aggregation chimeric embryos show that digynic cells, having only maternally derived chromosomes posses a different developmental potential than those having only paternally derived chromosomes (diandric cells) (13,28,29,30). Various studies have shown that this parental effect is restricted to certain chromosomal regions (31). It appears that (certain) homologous alleles from such imprinted regions have a different level of expression, depending on their parental origin (32-36). This phenomenon is called genomic imprinting, an epigenetic modification, which is germ line specific and maintained during fertilization and subsequent cell division. The biparental imprint pattern, as present in the zygote, has to be changed into a uniparental pattern before the final maturation into the mature gamete (14). When precisely erasement and the establishment of the new imprinting take place is not known. Neither is the molecular mechanism although it is evident that methylation is directly or indirectly involved (18,37).

Abnormal imprinting of the genome is involved in various human non-neoplastic and neoplastic conditions (10-12,38,39). Especially TGCTs are interesting in this context. The pathogenesis of these tumors starts most probably during embryonal development, affecting an early germ cell somewhere in its differentiation from a primordial germ cell to a gonocyte (1,5). In addition, the developmental potential of this common precursor CIS shows analogy with the induced murine digynic and/or diandric cells (13), or a combination of them. TGCTs can be composed of SE, showing similarities with primordial germ cells, and/or NS, showing embryonal and/or extraembryonal tissues (6). A linear progression model is suggested with SE as a stage in between CIS and NS (23,40,41). This shows striking analogy with reprogramming of mouse primordial germ cells to pluripotent embryonic stem cells (42).

TGCTs are characterized by a more or less specific chromosomal constitution (40). The significant and consistent underrepresentation of chromosome 15 in NS compared to SE, detected by karyotyping as well as by interphase cytogenetics on tissue sections (43) is one of these findings. This suggests that the copy number of chromosome 15 plays a

role in the progression from CIS to either of these histological subtypes. Our molecular data (Figure 1 and Table 2) support this hypothesis, showing overrepresentation of the whole chromosome 15 only in SE and loss in both SE and NS. Because of the percentages of allelic imbalances on this chromosome, we conclude that a disturbance in activity of a tumor suppressor gene localized on this chromosome is not crucial in the pathogenesis of TGCTs.

Alternatively, chromosome 15 might be involved due to the non-equivalence of certain genes on this chromosome depending on their parental origin (44,45). Recently a parental origin specific methylation polymorphism on chromosomal region 15q11-q13 has been reported (22). This allows the distinction between a biparental and uniparental (paternal or maternal) methylation status of cells which contains both parental regions. Because of the role of methylation in establishment of genomic imprinting (18,37), we used this methylation polymorphism on chromosome 15 as a tool to study whether TGCTs are uni- or biparentally imprinted. As expected, the controls, i.e., peripheral blood samples and testis parenchyma without spermatogenesis, show a biparental methylation pattern. This was also found for all NS studied, independently of the histological constitution. In contrast, DNA samples purified from testis with spermatogenesis show mainly the paternal methylation pattern. A spectrum between these two extremes was found for SE. Two showed a biparental methylation pattern, while partial demethylation was present in two others, possibly related to allelic imbalances. The basis for the disconcordance between the *Hpa*II and *Hha*I data found in four SE is unknown, but might be due to a disturbance of *cis* acting factors as recently suggested for Angelman syndrome patients (46). Intruiging is the finding of a (de)methylation pattern more similar to that found in DNA isolated from testis parenchyma with spermatogenesis in most of the SE, without allelic imbalances affecting this chromosomal region. The specific finding of demethylation for a subpopulation of SE is in agreement with published data concerning regions on both the X chromosome and some autosomal regions (21). Interesting is case G010, showing a relative overrepresentation of the maternal allele in the tumor, and at the same time a paternal-like methylation pattern. This indicates that the uniparental methylation pattern found in some SE is not influenced by the parental origin of the involved chromosomal region. Different degrees of methylation have been reported in various stages of the differentiation of the primordial germ cell to the mature gamete (20,47). These data suggest that primordial germ cells are unmethylated in both sexes, and that subsequently methylation occurs at specific sites depending on the sex on the individual. If this is true, our data could be explained in two ways. Either TGCTs originate from an early (primordial?) germ cell which still contains a biparental (pre-erased) imprinting, or they derive from a demethylated (erased) cell, and develop a biparental-like methylation pattern during tumor evolution. Alternatively, the demethylation of SE could be related to progression of the tumor.

Currently these tumors are studied using the PW71 probe (48), an additional tool to study the parental dependent methylation status of chromosomal region 15q11-q13.

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Chapter IX.

ORIGIN AND BIOLOGY OF A TESTICULAR WILMS' TUMOR.

Genes, Chromosomes & Cancer (in press).

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ABSTRACT

A pure triphasic testicular Wilms' tumor, without teratomatous elements, was studied using multiple techniques. Carcinoma in situ (CIS), the characteristic precursor of testicular germ cell tumors of adults (TGCTs), was found in the adjacent parenchyma. Flow cytometric analysis showed a single hypotriploid tumor stem line. Karyotyping of the tumor revealed some numerical and structural abnormalities, including an i(12p), the chromosomal marker of TGCTs. *In Situ* hybridization supported the karyotypic findings, and showed a similar numerical distribution in CIS and the tumor. Molecular analysis of the tumor illustrated that all short arms of chromosome 12, including i(12p), were of maternal origin. No 12q deletions were detected. In spite of complete loss of the paternal 11p13 band, the zinc finger regions and exons 2 and 6 of the *WT1* gene contained no aberrations. Therefore, this tumor suppressor gene is not inactivated due to aberrations in the studied regions. In addition, all four *WT1* alternative transcripts were expressed in the tumor. No aberrations were found on chromosomal band 11p15.5, 16q22.1 and 16q24. Both parental alleles of the human imprinted genes *H19* and *IGF2* were expressed in the tumor. This is the first report on the chromosomal and molecular characterization of an extrarenal Wilms' tumor. Its germ cell origin was unequivocally demonstrated.

INTRODUCTION

A germ cell origin of extrarenal Wilms' tumors, which show more frequently teratomatous (nonseminomatous (NS)) elements than renal Wilms' tumors (Magee et al., 1992; Fahner et al., 1993, and references cited therein), is hard to prove. This paper reports a multidisciplinary study of a Wilms' tumor, localized within an adult testis, which might give a clue as to its origin.

Its possible germ cell origin was studied by analysis of the pheno- and genotypical features that characterize testicular germ cell tumors of adults (TGCTs), which are known to be of germ cell origin (Damjanov, 1993). These malignancies have carcinoma in situ (CIS) in the adjacent parenchyma (Jacobsen et al., 1981; Skakkebæk et al., 1987), and a DNA content in the triploid range (Oosterhuis et al., 1989; De Graaff et al., 1992). Chromosome 12 is consistently involved, mainly as i(12p) (Murty et al., 1992; Geurts van Kessel et al., 1993). In addition, a role of chromosomes 15 and 22 has also been suggested (De Jong et al., 1990).

Deletions of chromosomal band 11p13 and/or 11p15.5 in TGCTs have been reported in several studies (Radice et al., 1989; Lothe et al., 1989; Looijenga et al., 1994), preferentially affecting the paternal allele (Lothe et al., 1993). While the incidence is in the same range to that reported in renal Wilms tumors (Slater and Mannens, 1992, for review), the latter show specific loss of the maternal 11p15.5 band, whereas for 11p13 sufficient data are not yet available (Reeve et al., 1984; Schroeder et al., 1987; Huff et al., 1990). From the 11p13 region, the Wilms' tumor 1 (*WT1*) tumor suppressor gene has been isolated and characterized (Madden et al., 1993 for review). Aberrations within this gene have been reported in sporadic and hereditary renal Wilms' tumors (Pelletier et al., 1991; Little et al., 1992; Slater and Mannens, 1992; Coppes et al., 1993; Haber et al., 1993), while no aberrations within the zinc-finger regions or exons 2 and 6 are found in TGCTs (Looijenga et al., 1994). In Wilms' tumors of Denys Drash patients, certain alternative *WT1* transcripts may be absent (Koenig et al., 1993).

Some renal Wilms' tumors show deletions of loci on the long arm of chromosome 16 (Huff et al., 1992; Maw et al., 1992). No data are available concerning the involvement of these loci in TGCTs.

Loss of uniparental expression, referred to as loss of imprinting (LOI), has been

reported for the *H19* and *IGF2* genes in sporadic renal Wilms' tumors (Rainier et al., 1993; Ogawa et al., 1993). Recently, we found a consistent biallelic expression of both genes in TGCTs (Van Gurp et al., 1994).

We here describe now a Wilms' tumor localized within the testis studied cytogenetically. In addition, parts of chromosomes 11, 12, 15, 16, and 22 were screened in more detail for allelic imbalances. For the chromosome 16 analysis a series of 33 primary TGCTs was included as reference. Possible aberrations within the zinc-finger regions and exons 2 and 6 of the *WT1* gene were also investigated. Expression of the different alternative splice variants of *WT1* was studied, as was possible LOI of the *H19* and *IGF2* genes.

MATERIAL AND METHODS

Case Description

A 30-year-old Dutch man presented with a solid painless testicular mass 20 cm in diameter, which had been present for approximately eight months. No elevated serum levels of alpha-feto protein (α-FP) and human chorio-gonadotropic hormone (hCG) were detected. The lactate dehydrogenase type 1 (LDH) was slightly elevated, i.e., 314 U/L. After orchidectomy, representative samples from the tumor and the adjacent testis parenchyma were snap frozen and fixed in formalin (4%) for paraffin embedding at the operation theater. Fresh tumor tissue was collected in culture medium (DMEM/HF12) containing 10% fetal calf serum (Gibco, Bethesda). The tumor was histologically classified according to the WHO classification (Mostofi et al., 1987). Immunohistochemistry was performed as described previously (Oosterhuis et al., 1989). After orchidectomy, a CT scan of the thorax and abdomen revealed no metastases and the patient received no further treatment. Twenty-five months after orchidectomy, the patient is still disease free.

Single Parameter DNA Flow Cytometry

DNA measurements were carried out on representative samples of the tumor by flow cytometry (FCM) using primary isolated ethanol (70% w/v,-20°C) fixed suspensions, and on cells isolated from paraffin embedded tissues as described (Hedley et al. 1983) with some minor modifications (Weaver et al. 1990). The histological composition of the samples for FCM was checked with hematoxylin and eosin stained tissue sections. Analysis was performed on a FACSCAN flow cytometer (Becton Dickinson, Mountain View, California), with interface and "Consort 30" data acquisition (Becton & Dickinson).

Karyotype Analysis

Fresh tumor tissue was minced and disaggregated with collagenase D (1 mgram/ml, Boehringer Mannheim) overnight at 34°C, whereafter it was seeded in extracellular matrix coated T75 flasks (Eldan, Israel) and incubated at 34°C in a humidified atmosphere with 5% CO_2 in air. The cells were harvested after four days of culture (Gibas et al., 1984) after 5 hours of colcemid exposure (Gibco, Bethesda), in a final concentration of 0.05μg/ml. Chromosomal preparations were made by conventional methods and G banding was performed according to a slightly modified method (Wang et al., 1972). The karyotype was described according to the ISCN 1991 guidelines (Mitelman, 1991).

In Situ **Hybridization (ISH)**

Centromere specific repetitive DNA probes for the chromosomes 1, 12, 15 and 22, designated pUC 1.77 (Cook et al., 1979), pα12H8 (Looijenga et al., 1990), D15Z1 (Higgens et al., 1985) and D22Z3 (McDermid et al., 1986), respectively, were used under high stringency hybridization conditions. The simultaneous application of immunohistochemistry (IHC) and ISH on frozen tissue sections of 20 micron thickness, as well as the screening and interpretation was performed as described (Looijenga et al., 1993).

Southern Blot Analysis

After written informed consent, heparinized peripheral blood samples of the patient and his parents were collected for isolation of control DNA. Southern blot analyses were performed using the same approach as reported (Looijenga et al., 1994). DNA loading variation was checked by visual judgement of ethidium bromide stained gels, as well as by using probe *IGH* for quantitation of loading variation and imbalances (Croce et al., 1979). This probe did not show a high frequency of allelic imbalances in a large series of TGCTs, including the cases studied here (Looijenga et al., unpublished results).

The following probes, in detail described elsewhere (Human Gene Mapping, 1991), were used: chromosome 8: *MYC*; chromosome 11: *CAT*, D11S417, *WT33*, D11S323, D11S324, D11S325, D11S16, D11S151, *FSHB*, *HRAS*, *IGF2*, *INT2*, *BCL1*; chromosome 12: *KRAS*, F8VWF, *GAPDH*, D12S6, D12S4, *IGF1*; chromosome 15: D15S2, *IGF1R*, *FES*; chromosome 16: *HP2A*, D16S127, D16S79, D16S7; chromosome 22: D22S181, D22S182, D22S183, D22S193, D22S201, D22S10.

The series of 33 primary TGCTs analyzed for involvement of chromosome 16 (this study) was described previously (Looijenga et al., 1994).

Polymerase Chain Reaction (PCR) and Single Stranded Conformation Polymorphism (SSCP) and Sequencing

The amplification by PCR of the zinc finger regions and exons 2 and 6 of the *WT1* gene, the SSCP, and the sequencing were performed as described (Looijenga et al., 1994). All PCR products showing possible mobility differences compared with a non-mutated control by SSCP were sequenced.

Reverse Transcription Polymerase Chain Reaction of the *H19* and *IGF2* Genes

The analysis was performed as described previously (Tadokoro et al., 1991; Zhang et al., 1992). Briefly, using PCR amplification and subsequent endonuclease digestion with *Alu*I and *Rsa*I or *Apa*I (Boehringer Mannheim GMBH, Mannheim, Germany), the blood sample of the patient was studied for heterozygosity of the *H19* and *IGF2* gene, respectively. After reversed transcription treatment and subsequent PCR on tumor isolated RNA the amplification products were digested as described above. The results were only interpreted when the negative control (without reverse transcriptase) lacked amplification products.

Reverse Transcription Polymerase Chain Reaction of the four alternative *WT1* Transcripts

Synthesis of cDNA was done according to the protocol reported above for the expression analysis of *H19* and *IGF2*. Using the primer P1-P4 (kindly provided by Dr. B. Royer-Pokora, Institut für Humangenetik, Heidelberg, Germany), the analysis of expression of the different splice variants of the *WT1* gene was performed as described (Brenner et al., 1992).

RESULTS

Grossly and histologically the tumor was confined to the testis. Microscopy showed a uniform pattern of blastema, epithelial, and stromal components (Figure 1A). Immunohistochemistry supported the diagnosis of a classical triphasic Wilms' tumor, and no expression of α-FP or hCG. The adjacent parenchyma contained seminiferous tubules with the typical histology of CIS (Figure 1B).

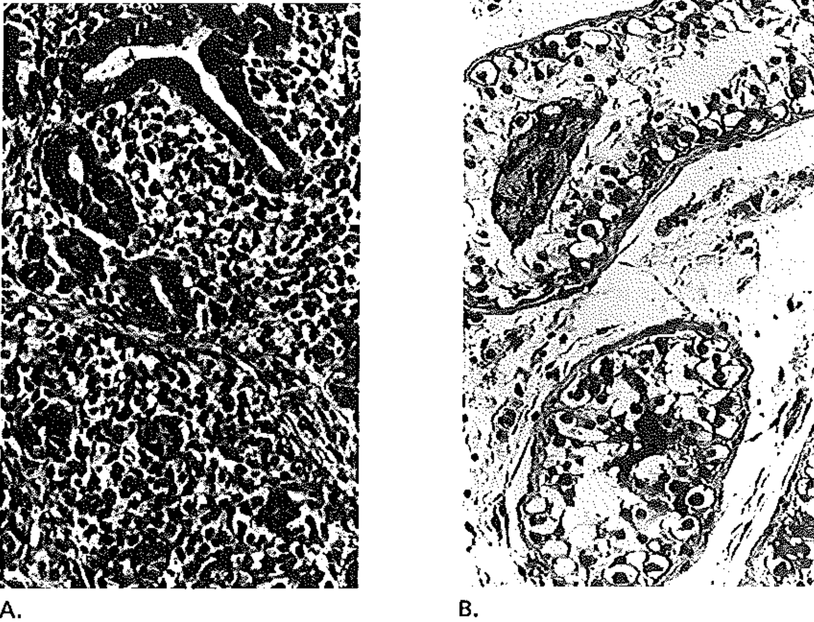

Fig. 1. Representative examples of (A) the tumor, and (B), the testis parenchyma stained with hematoxylin and eosin. Note the characteristic triphasic constitution of the tumor, as well as the presence of carcinoma in situ in the tubuli seminiferi.

The tumor showed a single stem line with a DNA Index of 1.25. Complete analysis of four and partial analysis of two tumor cells was possible, with a composite karyotype as follows: 54-57, X, -Y, +add(1)(p13), -2, +add(7)(p21), +8, +add(12)(p11), +add(12)(q14), +i(12)(p10), +add(16)(q13), +21, +mar1, +2-7 mar [cp 4] (Figure 2).

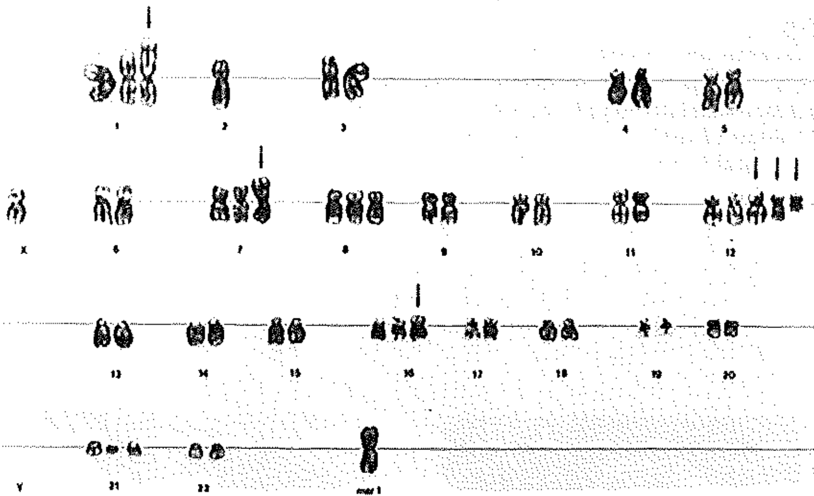

Fig.2. The composite karyotype of the tumor with the following chromosomal constitution: 54-57, X, -Y, +add(1)(p13), -2, +add(7)(p21), +8, +add(12)(p11), +add(12)(q14), +i(12)(p10), +add(16)(q13), +21, +8mar [cp 4].

To verify whether these data represented the tumor in vivo, ISH on tissue sections was performed. The results of this numerical analyses of the malignant cells are summarized in Figure 3.

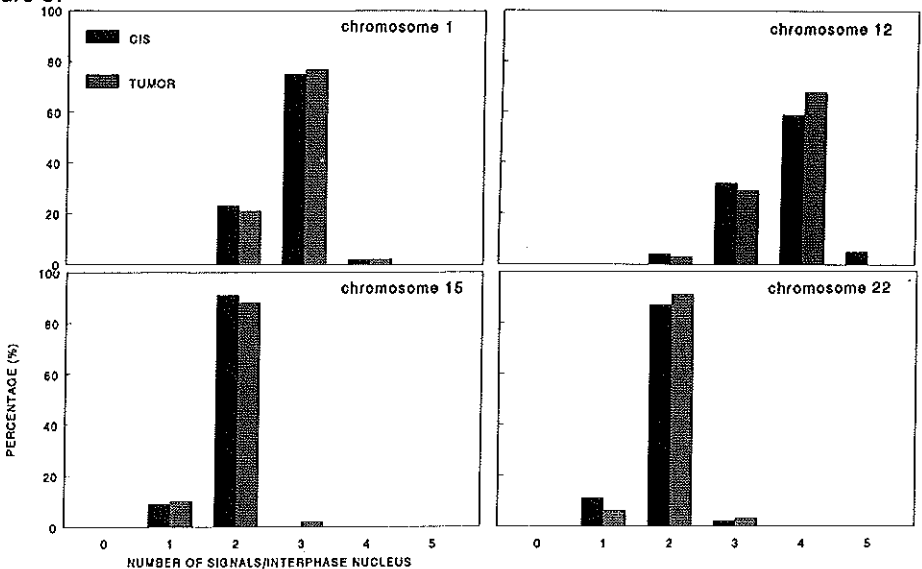

Fig.3. Summary of the in situ hybridization data for the numerical distributions analysis of centromeric regions of chromosome 1, 12, 15 and 22.

As expected from the cytogenetic analysis, mainly two signals per nucleus were found representing the chromosomes 15 and 22. The three signals for the centromeric region of chromosome 1 is concordant with the presence of the add(1)(p13). Four signals representing the centromeric regions of chromosome 12 were found. For all these chromosomes the adjacent CIS showed a virtually identical numerical pattern to that of the invasive tumor.

The results of Southern blot analysis of the tumor are summarized in Figure 4. In agreement with the karyotypic analysis, no aberrations were found on the long arm of chromosome 11, 15, 16, and 22. The long arm of chromosome 8 showed an overrepresentation, in agreement with the trisomy 8 found by cytogenetic analysis. A complete loss of the paternal short arm of chromosome 12 was present, while both maternal and paternal long arms of chromosome 12 were retained. This indicates that both normal chromosomes 12, the add(12)(q14), and the i(12p) are all of maternal origin, and that 12q sequences must be hidden in at least one marker. The add(16)(q13) was of paternal origin. The absence of imbalances on the long arm of this chromosome is in agreement with the cytogenetic findings.

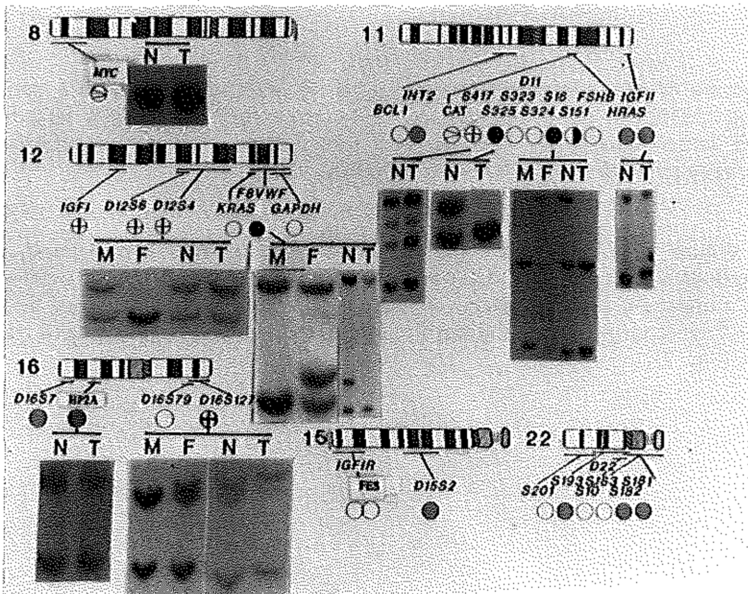

Fig.4. Summary of the molecular data on the testicular Wilms' tumor. ○ = not informative; ◍ = informative, no allelic imbalances; ⊕ = uniparental overrepresentation; ⊖ = overrepresentation, not informative ; ◐ = partial loss of heterozygosity; ● = complete loss of heterozygosity

The general involvement of chromosome 16 in a series of primary TGCTs is summarized in Figure 5. Two NS showed probable loss of the whole chromosome 16 (cases G017 and G031), whereas only 16q24 deletions were found in three SE (cases G011, G024, and G030) and two NS (cases G016 and G021). One SE showed LOH on 16q22.1 (case G012). In total, 7% of the SE and 6% of the NS showed overrepresentation of 16q sequences, and the figures for LOH are 27% and 22%, respectively.

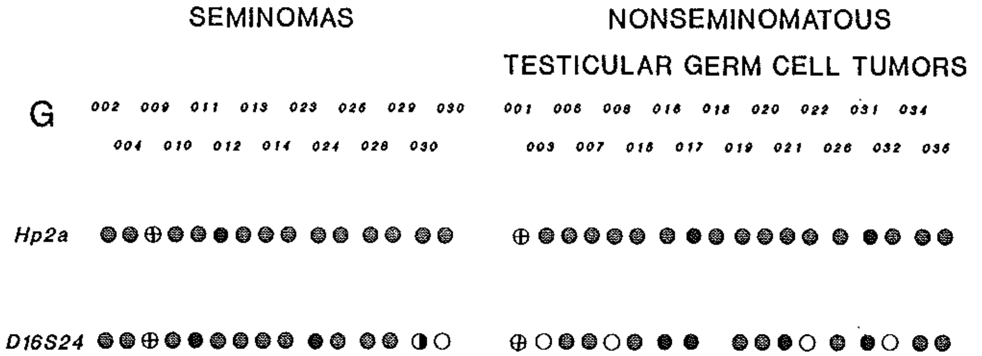

Fig.5. Summary of the allelic imbalances found on chromosome 16 in a series of 33 primary testicular germ cell tumors. ○ = not informative; ◍ = informative, no allelic imbalances; ⊕ = uniparental overrepresentation; ◗ = partial loss of heterozygosity; ● = complete loss of heterozygosity.

The Wilms' tumor showed complete loss of the paternal 11p13 band, while the maternal region was retained. No deletions and/or mutations were found within the *WT1* gene in the tumor. All possible splice variants were expressed (see Figure 6). Both the *H19* and *IGF2* were informative, and both parental alleles of both genes were transcripted in the tumor (Fig. 7).

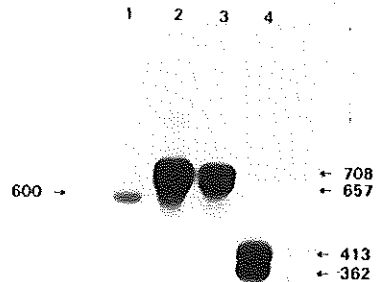

Fig.6.
Results of expression analysis of the different WT1 alternative splice variants using reverse transcription polymerase chain reaction amplification.

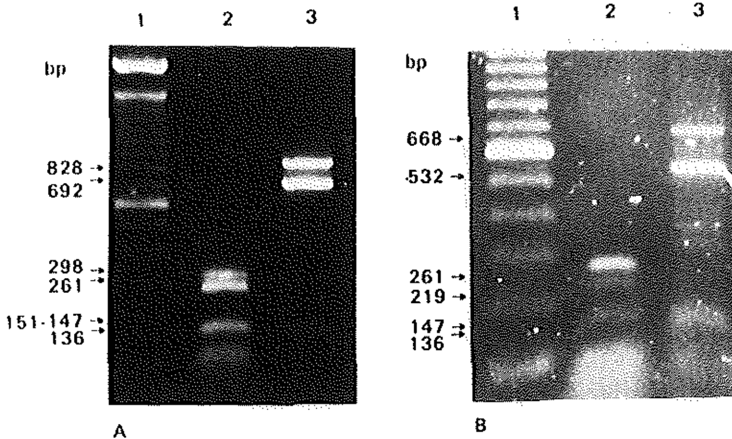

Fig.7. Results of the polymerase chain reaction amplification approach for the study of the mono- or biallelic expression of the H19 gene. Shown are the (A) DNA and (B) RNA data; lane 1 represents a 100 base pairs ladder, lane 2 the Alu-I digestion and lane 3 the Rsa-I digestion of the amplification products after electrophoresis. Note the presence of three amplification products in both the DNA and RNA analysis.

DISCUSSION

Wilms' tumors of the kidney are generally thought to arise from metanephric blastema. Most (80%) are diagnosed before the age of five years. They may be composed of three distinct differentiation lineages, i.e., blastema, stromal, and epithelial cells (Beckwith, 1986). Three loci may be involved in tumorigenesis by loss of tumor suppressor activity (Huff et al., 1992; Maw et al., 1992; Slater and Mannens, 1992). Aberrations within the *WT1* tumor suppressor gene, localized in chromosomal band 11p13, have been reported in renal Wilms' tumors (Little et al., 1993; Slater and Mannens, 1992; Coppes et al., 1993; Haber et al., 1993).

In contrast to renal Wilms' tumors, little is known about the origin and molecular mechanism(s) involved in the development of extrarenal Wilms' tumors. They show some similarities to their renal counterparts (Fernandes et al., 1989), but develop at a later age (Sahin et al., 1988), and show a higher incidence of NS elements (Kim et al., 1990 for review). These so-called "teratoid" Wilms' tumors are suggested to be of germ cell origin. TGCTs, the most common cancer in young men (Swerdlow, 1993), are derived from the neoplastic counterpart of primordial germ cells (Oosterhuis and Looijenga, 1993). Wilms' tumor like elements have been reported in extragonadal teratomas in childhood (Ward et al., 1974; Kim et al., 1990; Park et al., 1991). We have seen them in a residual mature teratoma, after polychemotherapy of a testicular NS (unpublished observation).

In contrast to the published Wilms' tumors associated with the gonads (Orlowski

et al., 1980; Sahin et al., 1988, and references therein), this Wilms' tumor was completely confined to the adult testis. The presence of CIS in the testicular parenchyma strongly suggests its germ cell origin (Skakkebæk et al., 1987), in agreement with the hypotriploid DNA content (Oosterhuis et al., 1989). In contrast, Wilms' tumors are predominantly near-diploid (Wang-Wuu et al., 1990; Slater and Mannens, 1992) and sometimes show an overrepresentation of structurally normal chromosomes 12 as sole chromosomal abnormality. The germ cell origin of the present tumor is confirmed by finding an isochromosome 12p (Atkin et al. 1982).

The similar ISH results of the CIS and invasive tumor, suggest a close evolutionary relationship between them, concordant with earlier data (Looijenga et al., 1993). The discrepancy between the numbers of centromeric regions of chromosome 12 detected by ISH (four) and karyotyping (five) may be due to the fact that only a minority of analyzed metaphases (two out of six) showed the add(12)(q14). A positive selection of these cells might occur during in vitro culturing. The uniparental origin of i(12p) in TGCTs (Peltomäki et al. 1992; Sinke et al. 1993) is concordant with the molecular findings in this Wilms' tumor. No allelic imbalances were present on 12q, 15q, 16q and chromosome 22. Cytogenetic analysis revealed a non-clonal loss of chromosome 15, supported by ISH. The relatively low percentage (15%) is probably the reason for the failure to detect allelic imbalances on this chromosome. The low frequency of aberrations on the long arm of chromosome 16 in primary TGCTs suggests that these regions are not consistently involved in the pathogenesis by loss of tumor suppressor gene activity.

Like some TGCTs (Lothe et al. 1993), this particular Wilms' tumor showed a complete loss of the paternal 11p13 region. Similar to all TGCTs (Looijenga et al., 1994), and most renal Wilms' tumors (Brown et al., 1993; Cowell et al., 1993) no aberrations within the zinc-finger regions or exons 2 and 6 of the *WT1* gene were found. Based on *WT1* expression data, this Wilms' tumor shared common features with Wilms' tumors of the kidney (Miwa et al. 1993) and of the endometrium (Roberts et al. 1993). All four *WT1* splice variants are expressed in the tumor. Therefore, a disturbed regulation of *IGF2* gene expression (Drummond et al., 1992) seems unlikely, but cannot yet be ruled out. This Wilms' tumor also showed, similar to TGCTs (Van Gurp et al., 1994), biallelic expression of both the imprinted genes *IGF2* and *H19*. Whether this is due to LOI or to an intrinsic characteristic of the cell of origin, is still unknown. These data do not exclude that *WT1* is involved in the development of Wilms' tumors and TGCTs, including the tumor we describe.

In conclusion, this multidisciplinary study unequivocally shows that this Wilms' tumor of the adult testis was of germ cell origin. This implies that primary or metastatic "germ cell tumor" has to be included in the differential diagnosis of extrarenal tumors of adults showing Wilms' tumor components with or without NS elements.

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Chapter X.

GENERAL DISCUSSION.

Paragraph 1.

INTRODUCTION.

In this chapter the results of the separate papers will be jointly discussed in relation to the three topics outlined in the introduction to this thesis. Briefly, what is the contribution of the results to the understanding of: the pathogenetic relationship between CIS, SE and NS (Chapter III and IV); the molecular mechanisms causing TGCTs (Chapters V, VI and IX); and the role of genomic imprinting in the pathogenesis of TGCTs (Chapters VII, VIII and IX). Finally taking into account the new data, we shall propose a further elaboration of our current pathogenetic model of TGCTs.

Paragraph 2.

RELATIONSHIP BETWEEN CIS, SE AND NS.

As pointed out in the introduction, light microscopy, ultrastructure, immunohistochemistry and ploidy of CIS- and SE cells are similar. Both are considered the neoplastic counterparts of primordial germ cells. No differences have been identified between CIS adjacent to SE and NS. These data are the basis of the now generally accepted view that CIS is the common precursor of SE and NS. If CIS cells progress to invasion without changing phenotype the result is SE. Alternatively, if the CIS cells do change phenotype when they become invasive the result is NS. What the mechanistic basis is of the change in phenotype is one of the unanswered questions in this model. The alternative model holds that SE and NS have different precursors with inherently different developmental potentials. The unsolved problem of this model in the phenotypic identity of CIS adjacent to SE and NS.

The results described in the Chapters III and IV throw new light on this problem. Using our probe pα12H8, specific for the centromeric region of chromosome 12 (Chapter II) and commercially available probes for the centromeric regions of chromosomes 1 and 15, we have compared numbers of centromeric regions of these chromosomes in SE, NS and their adjacent CIS.

The karyotypic data predict that SE contains a higher number of copies of chromosome 15 compared to NS. This result was indeed found using ISH. Surprisingly, we demonstrated that this numerical difference was not restricted to the invasive components, but also present in the adjacent CIS. This was the first demonstration of a difference between CIS/SE and CIS/NS. Interestingly, this is not accompanied by a phenotypic difference. It could mean that CIS/SE and CIS/NS have indeed different developmental potentials which become apparent only when the cells progress to invasiveness.

The second interesting finding was the similarity of the numbers of the chromosomes in CIS and its invasive counterpart, suggesting that both the non-invasive and invasive component within the same testis have undergone a partially similar karyotypic evolution. This finding was confirmed in Chapter IX where the numerical constitution of CIS was compared to that of the invasive pure Wilms' tumor of the testis.

In Chapter IV we have shown that most of the CTs do not obey the rules of the pure SE and NS with respect to their numbers of copies of chromosome 15. Some CTs have a low number of copies like NS, in the CIS, the SE and the NS component. Others have a high number of copies in the three components like SE. Some CTs behave like the pure SE and NS, with a high copy number in SE and its CIS, and a low copy number in NS and its CIS. These results suggest in the first place that there is more to determine the developmental potential of CIS than just the number of copies of chromosome 15. Secondly, the SE and NS components within CTs most often have a monoclonal origin. The NS component may be derived directly from CIS or the invasive SE. A minority of the CTs seem to be biclonal with the SE and NS component each derived from its own CIS.

As shown above the results of our ISH studies have contributed to our understanding of the relationship between CIS, SE and NS. On a side track three points raised in the literature have to be referred to in this paragraph.

As expected from chromosomal analysis of invasive SE and NS, two to three signals representing the centromeric regions of chromosome 1 were found per tumor nucleus. Depending on the sample studied, a majority of malignant cells (SE, NS and/or CIS) may contain one or two signals using this probe. We conclude therefore, that ISH with a chromosome 1 specific probe is inappropriate as only marker to identify invasive TGCT- or CIS-cells.

The pα12H8 shows 96.6% homology with pBR12 (1), and 92.4% with pSP12-1 (2), the other available chromosome 12 specific alphoid probes. These high percentages indicate that no differences in specificity of these probes can be expected (3). Using pα12H8 we could not confirm the consistent size difference between the signal of a normal chromosome 12 and an i(12p) reported with pSP12-1 (4,5).

Finally, we cannot support the contention that growth of SE in vitro selects for clones with i(12p). The number of ISH signals representing chromosome 12 centromeres in interphase nuclei is in complete agreement with the numbers of centromeres of chromosome 12 in karyotypes after direct harvesting. The numbers found with either of these techniques are the same as reported in SE karyotypes after culture in vitro.

Paragraph 3.

MOLECULAR MECHANISMS CAUSING GERM CELL TUMORS OF THE ADULT TESTIS.

Epidemiological data suggest that the initiation of TGCTs takes place in utero. In view of the relatively high incidence of bilaterality of this cancer perhaps in primordial germ cells, before they have finished their migration to the left and right gonadal blastema. Familial clustering of TGCTs suggests that a genetic predisposition (mutation?) may be transmitted through the germ line, without affecting normal development. This virtually excludes activated proto-oncogenes as candidates, because successful transmission of mutated proto-oncogenes through the germ line has not been reported.

Some investigators have claimed a role for loss of tumor suppressor genes localized on the long arm of chromosome 12. Their chromosomal and molecular analyses comprised a very small number of primary untreated TGCTs. We (Chapter VI) and others could not confirm their molecular finding of frequent deletions on 12q in TGCTs. It is

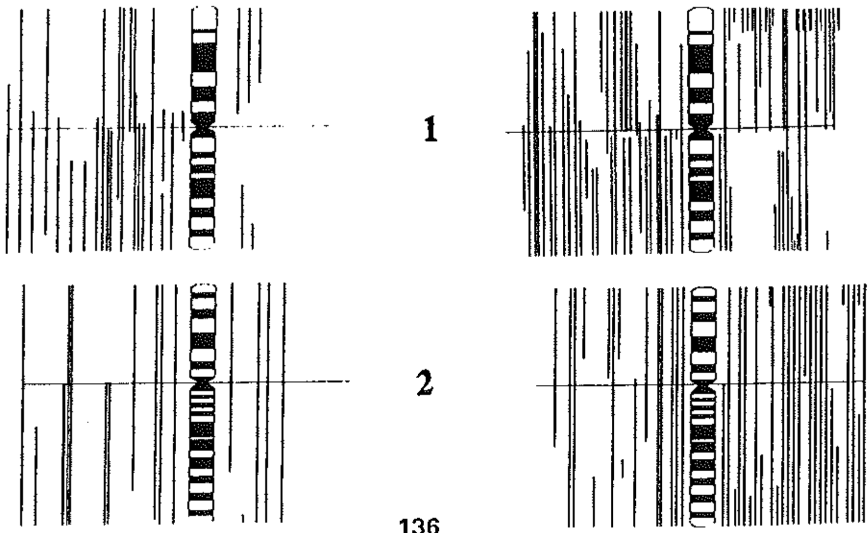
therefore unlikely that a tumor suppressor gene mapped on 12q plays an important role.

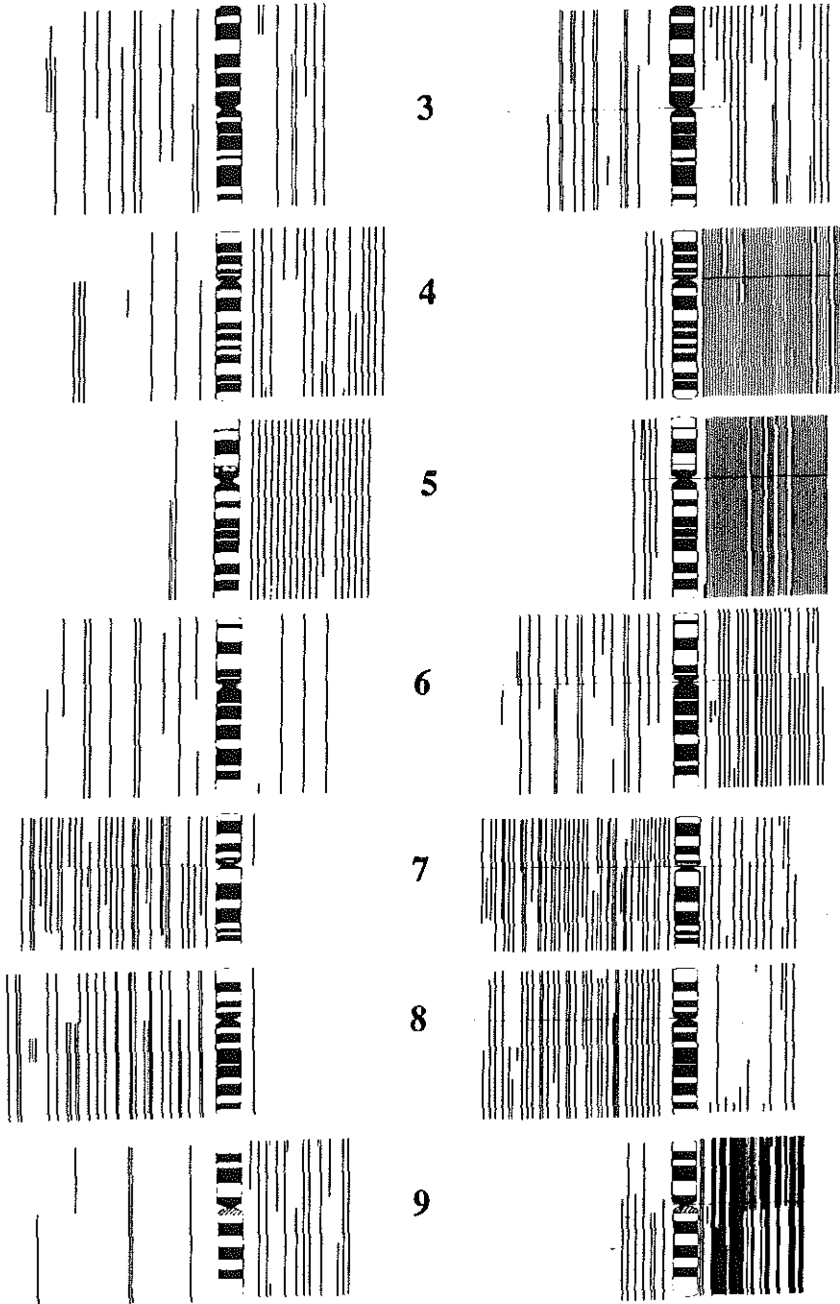
The chromosomes 11, 15, 16 and 22, which we have studied in more detail, show allelic imbalances in several regions, but deletions were rarely found in a higher percentage than 30%. This is the expected figure assuming random loss of (parts of) chromosomes from a near-triploid cell. The discrepancy between the chromosomal and molecular data on allelic imbalances on chromosome 22 suggests that during in vitro culture subclones with a lower number of copies of chromosome 22 are selected, or else that chromosomes 22 are lost during the procedure of culture and preparation of chromosome spreads. This illustrates, that the chromosomal data derived from samples which are consistently handled according to different protocols (SE and NS), must be verified by an approach excluding possible systemic errors.

In spite of frequent deletions of chromosomal band 11p13, no indications were found that inactivation of the tumor suppressor gene *WT*1 is involved in the pathogenesis of TGCTs. This is not surprising in view of the fact that WAGR syndrome and Denys Drash patients, whose genito-urinary abnormalities, including cryptorchidism, are caused by a mutation or a deletion affecting *WT*1, do not have an increased incidence of TGCTs. The inconsistent and low frequency of involvement of tumor suppressor genes and proto-oncogenes, confirmed by us, seems related to later stages of tumor progression and probably not to initiation. The higher age of patients with some of these changes than of those lacking them supports this view.

Two salient observations are the consistent aneuploidy of TGCTs and the overrepresentation of the short arm of chromosome 12, as i(12p) or otherwise. As pointed out in the introduction, polyploidization is probably an early step in the evolution of TGCTs, preceding the formation of i(12p) since heterozygosity of the long arm of chromosome 12 is retained.

TGCTs rarely contain *P53* mutations, and this probably results in a relatively stable genome (6, for review), explaining why homogeneously staining regions, and double minutes have never been documented in the large number of tumors that we have karyotyped. Karyotypic evolution in TGCTs is characterized by a steady net loss of chromosomes resulting in a predictable pattern of over- and underrepresentation, of which the overrepresentation of 12p is most conspicuous. This is schematically illustrated in Figure 6.

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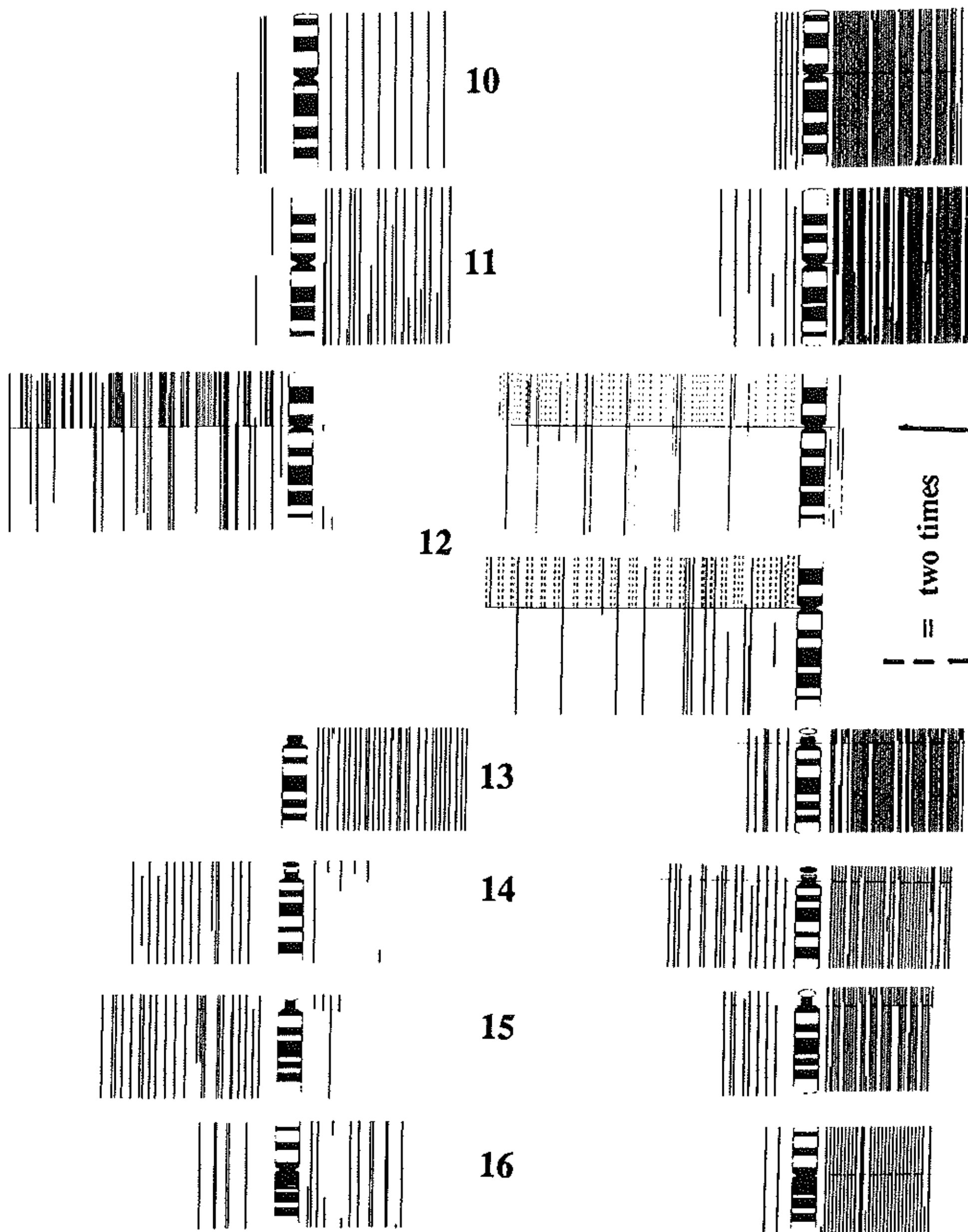
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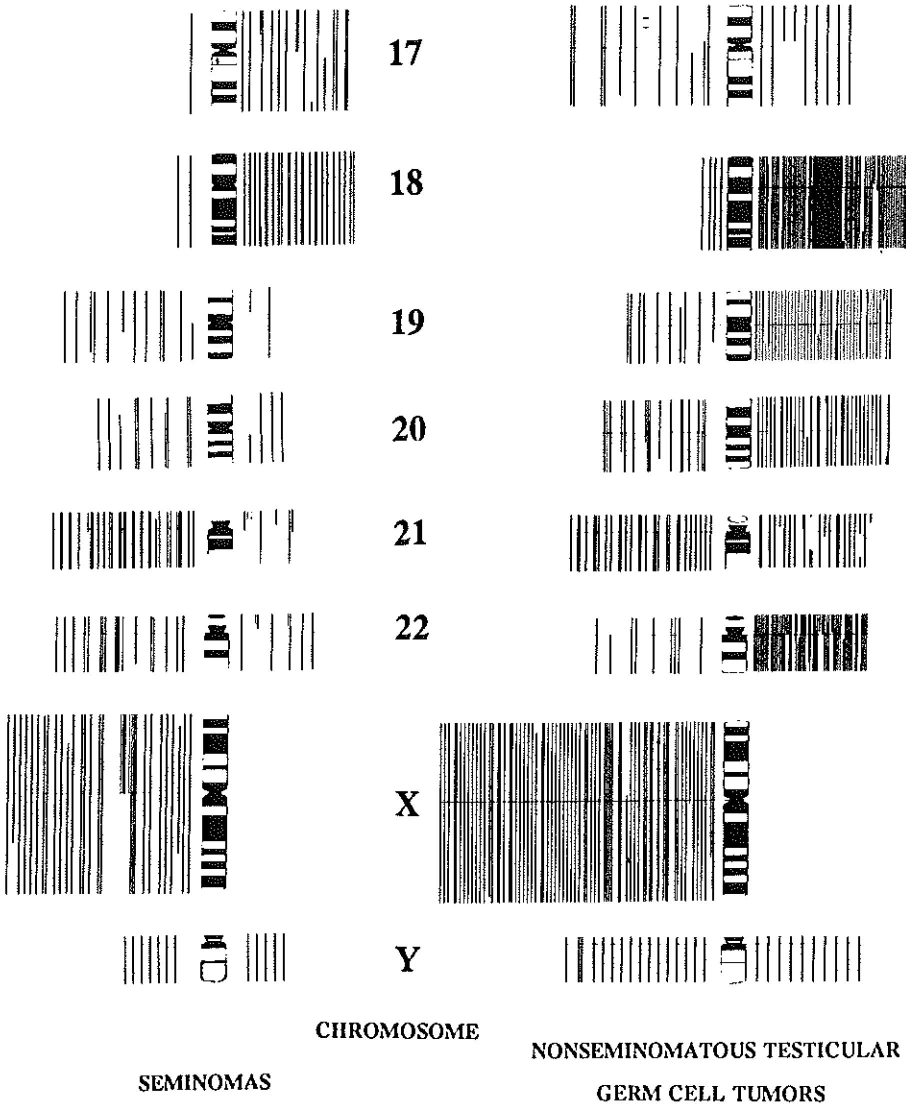

Fig. 6. Schematic representation of the over- and underrepresentation of (parts of) chromosomes in all karyotyped primary SE and NS by De Jong et al. The autosomes are calculated on the basis of a triploid DNA content (expected number is three), while the sex chromosomes are calculated on the basis of a diploid DNA content (expected number is one). The relative overrepresentated regions are indicated per tumor on the left sides of the chromosome, while the underrepresentated regions are indicated on the right side.

Paragraph 4.

GENOMIC IMPRINTING AND GERM CELL TUMORS OF THE ADULT TESTIS.

The suspicion that genomic imprinting might play a role in the pathogenesis of TGCTs is based on the assumption that the dermoid cyst of the ovary is the human counterpart of the mouse gynogenote, which favors somatic differentiation. Indeed the dermoid cyst of the ovary is exclusively composed of somatic tissues, and is the result of parthenogenetic activation of a postmeiotic diploid ovum, of which the chromosomes most likely have completed erasement and contain new female imprinting (7,8, for review). On the other hand the complete mole is the human counterpart of the mouse androgenote which favors trophoblast differentiation. The complete mole consists only of trophoblastic tissue and results from the fertilization of an empty ovum by two sperm. It has, therefore, an exclusively male imprint (7,8, for review).

TGCTs are derived from premeiotic primitive germ cells. It is not known when precisely in the development of the primordial germ cell to a mature sperm erasement takes place and the new uniparental pattern of imprinting is established. It is conceivable that TGCTs are derived from primitive germ cells in different stages of these processes, and that their developmental potential is more or less determined by the imprinting status of these cells.

We hypothesize that the pluripotent character of NS requires a biparental imprinting. The finding by Lothe (9) and us (Chapter VI) that the maternal copy of 11p and of chromosome 22 are preferentially retained in TGCTs suggests non-equivalence of the paternal and the maternal copy of chromosomes 11 and 22, compatible with a biparental imprinting pattern. Further support for this assumption comes from our results with the probe DN34 (Chapter VIII), which indicate retention of both the paternal and maternal methylation pattern of chromosomal region 15q11-q13. These findings imply that the primitive germ cell from which the NS is derived still has its original "somatic" imprint, and was not yet erased: a "pre-erased" precursor cell.

SE on the other hand, when probed with DN34 and PW71, may show a methylation pattern as NS, while the majority show a demethylated pattern, which is compatible with a paternal imprint. The fact that this pattern was even found on a maternally derived chromosome 15 suggests that the cell is post-erased, and acquired a fresh paternal imprinting. These results imply that most SE are malignant counterparts of cells which are in or even past the process of erasement and renewed imprinting. This might be related to the cell of origin, or acquired during progression.

The biallelic expression of the genes *H19* and *IGF2* in both SE and NS is not explained by the imprinting status of the presumed precursor cell (10). It may be due to a different regulation of imprinted genes in primordial germ cells than in somatic cells. Since we have used a qualitative RT-PCR technique we have no idea of the relative level of expression of the paternal and maternal allele. Therefore, leakiness of the imprinted allele cannot be excluded. Another possible explanation would be LOI as a result of tumor progression, as demonstrated in most Wilms' tumors for *IGF2*.

The on average hypertriploid DNA content of CIS and SE and the hypotriploid DNA content of NS, with highly consistent over- and underrepresentation of certain (parts of) chromosomes makes the situation even more complex. Paternally and maternally imprinted genes may lie on non-homologue chromosomes. Disturbed ratios between (parts

of) these chromosomes may result in mimicking of a paternal or a maternal pattern of genomic imprinting, and thereby favor either trophoblastic or somatic differentiation.

Paragraph 5.

PATHOGENETIC MODEL FOR GERM CELL TUMORS OF THE ADULT TESTIS: AN UPDATE.

When initiation of TGCTs affects a primitive germ cell prior to erasement, still bearing a biparental pattern of genomic imprinting, it may be activated -reprogrammed- to pluripotency. If the initiated cell is further down the road of maturation towards spermatogenesis (associated with loss of its pluripotent developmental potential (11), and, therefore, has undergone erasement and perhaps already new paternal imprinting, reprogramming to a pluripotent cell is less likely. The neoplastic cell will retain the phenotype of a primitive germ cell, resulting in SE.

It is challenging to try to combine this hypothesis on the role of genomic imprinting with our current model of the pathogenesis of TGCTs. An early, perhaps the initiating, event in the pathogenesis of TGCTs is polyploidization of a primitive germ cell. The tetraploid cell is more prone to mitotic errors. Due to this genetic instability, it may loose or gain chromosomes during progression. The net effect in TGCTs is reduction of the DNA content. The polyploid cells have survival and/or growth advantage over their normal counterparts which may be further enhanced by an increasing number of copies of (parts of) 12p.

These events, polyploidization, non-random loss of chromosomes and overrepresentation of 12p are apparently very similar for initiated pre-erased (early) primitive germ cells and the initiated erased (late) primitive germ cells. This is not amazing since both cell types grow as CIS in the seminiferous tubules, in the niche of spermatogonia, with tightly controlled growth conditions. The only difference thus far demonstrated between the two is a significantly higher number of copies of chromosome 15 in CIS/SE than in CIS/NS, and a lower copy number of centromeric regions of chromosome 12 in CIS/NS compared to CIS/SE. Their possible difference in developmental potential may only become apparent in the invasive stage. It is also only after the transition to invasiveness that the difference in ploidy between SE and NS starts to develop. Between early invasion and clinical presentation NS does loose additional chromosomes compared to the *in situ* stage, while SE does not. This may be directly related to the change in phenotype in NS upon invasiveness. Certain (parts of) chromosomes with genes coding for factors which are necessary for growth as phenotypically SE cells in the seminiferous tubule may become superfluous for a NS. This would not be the case for SE. The changes which do occur in CIS/NS as compared to CIS/SE, e.g., the extra loss of copies of chromosomes 15, may relate to characteristics of the early pre-erased primitive germ cell. Alternatively, the initiation of both SE and NS may affect a primitive germ cell at the same stage of maturation, while during progression of SE (in the *in situ* or invasive stage) further differentiation along the spermatogenic lineage takes place. This model is in principle similar to the one described, except that developmental potential determining progression occurs after initiation, while in the first

model it is intrinsic to the initiated cell.

We have presented the first and still limited evidence that CTs -with a SE and a NS component- may be derived from one CIS or in the minority of the cases from two in situ carcinomas. In the latter situation the two lineages occur simultaneously in one testis. When derived from a single primitive germ cell, it might in terms of maturation be the counterpart of the precursor of NS, which may differentiate more into the spermatogenic lineage in either the pre- or invasive stage. In contrast, CTs may also be derived from a precursor which is concerning its maturation stage in between the precursor of NS and SE. Finally, invasive SE cells, might be activated (reprogrammed) to pluripotency due to imbalances between (parts of) chromosomes, thereby mimicking a biparental pattern of genomic imprinting.

Our updated model is summarized in Figure 7. It combines elements of the two extremes, the linear progression model and the model that assumes separate precursor lesions for each histological subtype. Our current thinking is that NS and SE may originate from a similar kind of precursor, the primordial germ cell, but they may have already different developmental potentials. At the same time all NS originate from CIS of which the cells have a seminomatous phenotype, and NS may also stem from invasive SE through reprogramming.

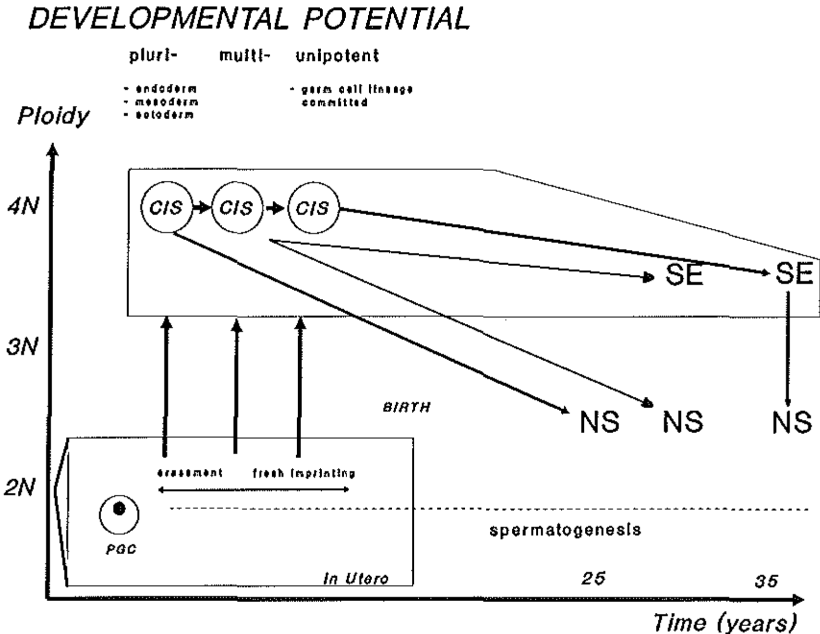

Fig. 7. *Pathogenetic model for germ cell tumors of the adult testis, with the developmental potential explained by the stage of maturation of the precursor cell, before or after acquisition of its invasive behavior. (PGC = primordial germ cell; CIS = carcinoma in situ, SE = seminoma, NS = nonseminomatous testicular germ cell tumor)*

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Chapter XI.

FUTURE RESEARCH.

Paragraph 1.

INTRODUCTION.

In our future research on TGCTs we will focus on two topics: the early events in the development of this cancer, that is the *in situ* stage, and the impact of genomic imprinting on its developmental potential.

Paragraph 2.

STUDY OF CIS.

The most important message from our studies on the pathogenetic relationship between CIS, SE and NS is that CIS present in orchidectomy specimens with an invasive TGCT is not representative for the early stage of this cancer. The ploidy and ISH data show that CIS adjacent to an invasive TGCT has undergone a karyotypic evolution which resembles that of the invasive component. The additional progression step has endowed the invasive cells with the capacity to survive and proliferate outside the micro-environment of the seminiferous tubules, associated with net loss of chromosomal material in NS. The early events in the development of TGCTs have to be investigated in CIS before it progresses to invasiveness.

Little is known about these early stages of development of TGCTs. Is their one common CIS for all TGCTs, or do CIS of SE and CIS of NS pursue their own course from the earliest stage onwards? Our ISH data, showing a different chromosomal constitution of CIS adjacent to a clinically manifest pure SE or NS, support a different karyotypic evolution of the CIS populations, but do not exclude a common origin. When does polyploidization take place in the development of CIS, and what is the mechanism. The detection of aneuploid germ cells without indication of malignancy in men with and without cryptorchidism (1,2) is interesting in this context. At what stage of development occurs the gain of the short arm of chromosome 12, and what is the biological function of it. Is the advantage due to overrepresentation of the whole p-arm or only a part of it? No candidate gene has been identified yet, but *PTHRP* (3), the gene encoding liver specific glycogen synthase (4) or cyclin (5) could be involved. The recently reported stimulation of proliferation of murine primordial germ cells by tumor necrosis factor-α (TNF-α), in the stage before and during their migration to the gonadal blastema (6) is interesting, because the *TNF*-receptor 1 is localized on chromosomal band 12p13 (7).

The study of these early stages of TGCTs is not only intriguing from a tumor biological point of view, but may also be clinically useful. For example a specific molecular marker might allow detection of TGCTs in a early stage of development, even before progression to invasiveness, when the condition is still easily cured (8).

Paragraph 3.

STUDY OF GENOMIC IMPRINTING.

In spite of being in its infancy, our study of the role of genomic imprinting in the development of TGCTs has already shown that the imprinted genes *H19* and *IGF2* behave differently in these tumors than in the normal tissues thus far studied. To investigate whether the biallelic expression of these genes in TGCTs is due to "leakage", as suggested in parthenogenetic murine embryos (9), needs a quantitative analysis. Unknown is how the expression of *H19* and *IGF2* relates to their methylation status in these tumors. Similar to its murine counterpart, the human silent (paternal) H19 gene is normally hypermethylated (10). Preliminary results of Southern blot analysis show that only one parental *H19* allele is demethylated in peripheral blood, while both are in TGCTs (illustrated in Figure 8).

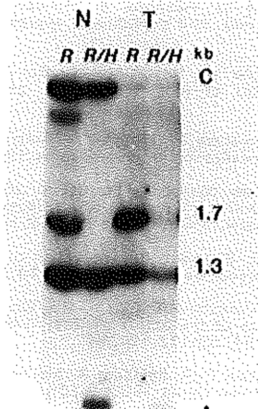

Fig. 8.
An example of Southern blot analysis of DNA isolated from peripheral blood and a NS, after digestion with RsaI alone (a marker for parental origin (11), or in combination with HpaII.

This study will be carried out in more detail for example using a ligation-mediated polymerase chain reaction (12). The fundamental question of how the imprinting of genes is regulated will be included. The regulatory role of *WT1* products on the expression of *IGF2* (13), possibly linked to *H19* (14,15) is interesting in this context. The possible biological significance of *WT1* in TGCTs is illustrated by the difference in incidence of a polymorphism within this gene in patients with bilateral TGCTs compared to those with unilateral TGCTs and normal controls (16). The expression pattern of the imprinted genes in TGCTs might be the result of several factors, e.g., the imprinting status of the precursor, loss of imprinting, the specific over- and underrepresentation of (parts of) chromosomes, possibly containing imprinted or imprinting (17) genes.

If we are right that the developmental potential of TGCTs correlates with genomic imprinting of its precursor, the study of this cancer will shed light on the mechanism and timing of erasement and subsequent re-imprinting during the maturation of a primordial germ cell to a mature gamete. If this difference in genomic imprinting is inherent to the cell of origin, or acquired during progression will be studied using CIS before and after progression to invasiveness. It is obvious that this study should not be limited to TGCTs. The ovarian germ cell tumors, as well as those of extragonadal localizations will be included. In addition, the other possibly imprinted genes, *SNRPN*, *IGF2*-receptor and *WT1*

(18-21), will be investigated, to fully exploit the potential of germ cell tumors in the study of genomic imprinting.

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Chapter XII.

Summary

Samenvatting

Summary.

Human germ cell tumors of the adult testis (TGCTs) are histologically and clinically divided into two entities: seminomas (SE), composed of cells which are considered the neoplastic counterparts of primitive germ cells (gonocytes) and nonseminomatous TGCTs (NS) of which pluripotent embryonal carcinoma cells (EC) are the stem cells. SE and NS may occur within a single testis, geographically separated or mixed. These TGCTs containing both components are called combined tumors (CTs). In a neoplastic caricature of normal development, these EC cells may produce a wide range of embryonic and extraembryonic tissues: immature and mature teratoma, yolk sac tumor and choriocarcinoma. The common precursor of both entities is carcinoma in situ (CIS). CIS cells are phenotypically identical to SE-cells.

In this thesis three aspects of the biology of these tumors are studied: the pathogenetic relationship of CIS, SE and NS, the molecular mechanisms causing TGCTs and the role of genomic imprinting in their development.

The pathogenetic relationship between CIS, SE and NS was studied using a combination of immunohistochemistry and interphase cytogenetics with centromeric region specific probes for the chromosomes 1, 12 and 15 on thick tissue sections. This approach allows numerical chromosomal analysis of specified cells within their histological context. Because of the consistent involvement of chromosome 12 in TGCTs, we wanted to include this chromosome in our study although a chromosome 12 centromeric region specific probe was not available at that time. Therefore, a chromosome 12 specific repetitive DNA probe suitable for in situ hybridization was isolated (Chapter II). Analysis of pure SE and pure NS, as well as their corresponding CIS revealed that both the invasive and non-invasive components showed a remarkably similar chromosomal constitution for the studied chromosomes (Chapter III). This indicates that the non-invasive cells progress through a similar kind of karyotypic evolution as the invasive cells. Therefore, we conclude that for identification of the early events in the pathogenesis of TGCTs, CIS before progression to invasiveness should be studied. Our in situ numerical chromosomal analysis with preservation of histology confirmed the overrepresentation of chromosome 15 in SE compared to NS, already indicated by conventional karyotyping. A significantly higher number of copies of chromosome 15 was also found in the CIS of SE compared to CIS of NS. This is the first demonstration of a difference between CIS adjacent to SE and NS thus far. We used the specific over- and underrepresentation of chromosome 15 as marker to study the clonality of SE and NS components within combined tumors (Chapter IV). Usually the CIS, SE and NS components of CTs had the same number of copies of chromosome 15, either high or low, suggesting that the SE and NS component were derived from a single CIS. The minority of the cases had a high copy number in the SE and its adjacent CIS and a low copy number in the NS and its adjacent CIS, suggesting independent origin of the SE and NS component.

Because deletions affecting the short arm of chromosome 11 have been suggested to be important in the development of TGCTs, we investigated this in more detail, with special attention to the role of the tumor suppressor gene *WT*1 (Chapter V). Deletions of band 11p13 and/or 11p15.5 occur relatively frequent in both SE and NS, but no indication was found that inactivation of *WT*1 was involved. Even in a unique pure testicular Wilms' tumor of germ cell origin (Chapter IX), no aberrations were detected in this gene, in spite of loss of the paternal 11p13 band. The molecular data on the presence of allelic imbalances of chromosomes 12, 15, and 16 in TGCTs (Chapter VI, VIII, IX) supported the chromosomal findings, and no specific hot spots for deletions, indicative for

the involvement of a tumor suppressor gene were found. The karyotypically found overrepresentation of chromosome 22 in SE compared to NS might be due to the different protocols applied: direct harvesting for SE and short term in vitro culture for NS (Chapter VI). In conclusion, the imbalances present in TGCTs are most probably related to net loss of (parts of) chromosomes from a polyploid precursor, and do not pinpoint a chromosomal region which is involved in the initiation of this cancer.

The histological diversity of TGCTs, showing a primitive germ cell-like phenotype (seminomas), or a phenotype reflecting embryonal and/or extra-embryonal differentiation (nonseminomatous TGCTs), suggests that the pathogenesis of this cancer is influenced by mechanisms like genomic imprinting involved in early embryogenesis. We studied the possible role of genomic imprinting by analysis of the expression of the imprinted genes *H19* and *IGF2*. In contrast to most of the tissues during normal development, TGCTs showed biallelic expression of both genes, independent of the histological composition (Chapter VII). Whether this is due to an inherent phenomenon of the cell of origin of this cancer, or to a consistent relaxation of genomic imprinting is unknown thus far. To investigate the pre- or post-erasement status of TGCTs, we analyzed the parent determined methylation status of chromosomal region 15q11-q13, detected by DN34. All NS showed a pattern indicative for the presence of both paternally and maternally imprinted chromosomes. In contrast, only a minority of SE contained this pattern. Most SE revealed retention of the paternal and a varying degree of loss of the maternal methylation pattern. This might indicate heterogeneity of SE in their maturation along the germ cell lineage, which could be related to their developmental potential, i.e., capacity to progress into NS. In conclusion, we suggest that the developmental potential of TGCTs, leading to pure SE, pure NS (with or without trophoblastic differentiation), or a mixture of it, is determined by the maturation stage of the cell of origin, which might be an inherent or acquired characteristic of the affected primitive germ cell. This is an exciting and testable hypothesis, which may teach us more about genomic imprinting and erasement, as well as the mechanisms involved.

Samenvatting.

Humane kiemceltumoren van de volwassen testis (TKCT) zijn histologisch en klinisch onder te verdelen in twee groepen: seminomen (SE), samengesteld uit cellen welke verondersteld worden de neoplastische tegenhangers te zijn van primitieve kiemcellen (gonocyten) en nonseminomateuze TKCT (NS) waarvan de pluripotente embryonale carcinoom (EC) cellen de stamcellen zijn. SE en NS kunnen voorkomen in één testis, geografisch apart of echt gemengd. Deze TKCT met beide componenten worden "gecombineerde tumoren" genoemd (CT). In een neoplastische karikatuur van de normale ontwikkeling, kunnen de EC cellen een breed spectrum van zowel embryonale als extra-embryonale weefsels vormen; immatuur en matuur teratoom, dooierzak tumor en choriocarcinoom. De gemeenschappelijke voorloper van beide tumor typen is carcinoma in situ (CIS). CIS cellen zijn phenotypisch identiek aan SE cellen.

In dit proefschrift zijn drie aspecten van de biologie van deze tumoren bestudeerd: de pathogenetische relatie tussen CIS, SE en NS, de moleculaire mechanismen betrokken bij het ontstaan van TKCT en de rol van genomische inprenting in hun ontwikkeling.

De pathogenetische relatie tussen CIS, SE en NS is bestudeerd met behulp van een combinatie van immunohistochemie en interfase-cytogenetica met centromeer-specifieke probes voor de chromosomen 1, 12 en 15 op dikke weefselcoupes. Deze benadering maakt numerieke chromosomale analyse van specifieke cellen in hun histologische context mogelijk. Op grond van de consistente betrokkenheid van chromosoom 12 in TKCT, wilden wij dit chromosoom betrekken in onze analyse. Omdat geen centromeer-specifieke probe voor chromosoom 12 beschikbaar was, hebben wij deze geïsoleerd (Hoofdstuk II). Bestudering van pure SE en pure NS, en de bijbehorende CIS, gaf in zowel de invasieve als de niet-invasieve componenten sterk overeenkomstige aantallen centromeren van de drie onderzochte chromosomen te zien (Hoofdstuk III). Dit suggereert dat de niet-invasieve cellen een vergelijkbare karyotypische evolutie doormaken als de invasieve cellen. Op grond hiervan concluderen wij dat voor de karakterisatie van de vroege gebeurtenissen in de pathogenese van TKCT, CIS voordat het progressie naar invasiviteit doormaakt, het meest geschikt is. Onze numerieke in situ chromosomale analyse in de histologische context bevestigt de oververtegenwoordiging van chromosoom 15 in SE ten opzichte van NS, zoals gevonden was met conventionele karyotypering. Bovendien werd een significant hoger aantal copieën van chromosome 15 gevonden in CIS behorende bij SE dan in CIS behorende bij NS. Dit is de eerste bevinding van een verschil tussen CIS behorende bij SE en CIS behorende bij NS. Wij hebben deze specifieke over- en ondervertegenwoordiging van chromosoom 15 gebruikt als criterium om de clonaliteit van SE en NS componenten van CT te bestuderen (Hoofdstuk IV). In de meeste gevallen vertoonde zowel de CIS, de SE als ook de NS component van CT een identiek aantal copieën van chromosoom 15, of hoog of laag, suggererend dat de SE en de NS component ontstaan zijn uit een enkele CIS. De minderheid van de gevallen had een hoog aantal copieën in de SE component en het bijbehorende CIS, en een laag aantal copieën in de NS component en het bijbehorende CIS, hetgeen een onafhankelijke origine van de SE en NS component suggereert.

Omdat in de literatuur is gesuggereerd dat deleties van de korte arm van chromosome 11 belangrijk zijn voor het ontstaan van TKCT, hebben wij dit gebied meer in detail bestudeerd, met bijzondere aandacht voor de rol van het tumor suppressor gen *WT1* (Hoofdstuk V). Deleties van de chromosomale regio 11p13 en/of 11p15.5 werden relatief frequent aangetroffen in zowel SE als NS, maar er was geen indicatie voor inactivatie van *WT1*. Zelfs in een unieke pure testiculaire Wilms' tumor van kiemcel-oorsprong

(Hoofdstuk IX), werden geen afwijkingen in dit gen aangetroffen, ondanks verlies van de vaderlijke regio 11p13. De moleculaire gegevens over allelische imbalansen van de chromosomen 12, 15 en 16 in TKCT (Hoofdstuk VI, VIII, IX) ondersteunen de chromosomale bevindingen. De cytogenetisch gevonden oververtegenwoordiging van chromosoom 22 in SE ten opzichte van NS kan het gevolg zijn van de verschillende behandelingen die toegepast zijn voor de karyotypering: direct oogsten voor SE en korte termijn in vitro kweek voor NS (Hoofdstuk VI). Concluderend stellen wij dat de imbalansen die aanwezig zijn in TKCT zeer waarschijnlijk het gevolg zijn van netto verlies van (delen van) chromosomen uitgaande van een polyploide voorloper, en geen indicatie geven voor een chromosomale regio die betrokken is bij het ontstaan van deze kanker.

De oorsprong -primitieve kiemcellen- en het histologisch spectrum, variërend van neoplastische kiemcellen bij SE en weefsels uit de (vroege) embryonale ontwikkeling bij NS, suggereren dat in de pathogenese van deze kanker mechanismen, zoals genomische inprenting, betrokken bij de normale embryogenese, van belang zijn. Wij hebben de mogelijke rol van genomische inprenting bestudeerd door middel van analyse van de expressie van de genen *H19* en *IGF2* die inprenting ondergaan. In tegenstelling tot de weefsels tijdens normale ontwikkeling, gaven de TKCT bialleIische expressie van beide genen te zien, onafhankelijk van de histologische samenstelling van de tumor (Hoofdstuk VII). Of dit het gevolg is van een intrinsieke eigenschap van de cel waaruit deze kanker ontstaat, of van verlies (relaxatie) van genomische inprenting is onbekend. Om na te gaan of TKCT ontstaan voor of na het wissen van de biparentale inprenting, is het parentaal bepaalde differentiële methylerings patroon van de chromosomale regio 15q11-q13 bestudeerd met de probe DN34. Alle NS vertoonden een biparentaal methyleringspatroon overeenkomend met de aanwezigheid van chromosomen die nog herkend kunnen worden zijnde afkomstig van de vader of van de moeder. SE daarentegen had dit patroon in een minderheid van de gevallen. De meeste SE gaven een vaderlijk methyleringspatroon te zien, met een wisselende mate van verlies van het moederlijke methyleringspatroon. Dit suggereert dat SE onderling verschillen in de mate waarin de tumorcellen zijn uitgerijpt in de richting van kiemcellen. Dit verschil in uitrijping is wellicht van invloed op het ontwikkelingsrepertoir van de SE cellen. Een verder uitgerijpte cel is beperkt in zijn repertoir en kan alleen nog maar neoplastische kiemcellen vormen (SE). De minder uitgerijpte cel kan nog worden geactiveerd (gereprogrammeerd) tot een pluripotent programma (NS). Concluderend: wij suggereren dat het ontwikkelingsvermogen van TKCT, resulterend in pure SE, pure NS (met of zonder trophoblastaire differentiatie), of een samenstelling hiervan, bepaald wordt door de uitrijping van de neoplastische primitieve kiemcel. Deze uitrijping wordt enerzijds bepaald door het ontwikkelingssstadium van de primitieve kiemcel waaruit de kanker ontstaat, anderzijds kan de neoplastische kiemcel tijdens ontwikkeling van de tumor verder uitrijpen, of worden gemodificeerd door nabootsing van inprenting.

Bestudering van deze boeiende, toetsbare hypothese kan ons inzicht vergroten in de pathogenese van kiemceltumoren en in de moleculaire mechanismen van het wissen en opnieuw aanbrengen van genomische inprenting.

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Zoals de polyploidizatie cruciaal is in het ontstaan van de kiemceltumoren van de volwassen testis (*dit proefschrift*), zijn mijn ouders dat, buiten het puur biologische aspect, voor mij geweest. Zij hebben op een aantal kritische momenten in mijn leven hun stem laten gelden. Mijn (soms te grote) eigenwijsheid heeft hen niet in de weg gestaan mij altijd te stimuleren en te steunen, waarvoor ik hen buitengewoon dankbaar ben. Dagelijks geniet ik van mijn liefde voor de muziek (een eigenschap verworven van mijn vader) en mijn onvermogen om dik te worden (een eigenschap verworven van mijn moeder). Terwijl het mij duidelijk maakt dat ik nog een relatief rustig leven leid, en dat de jaren gaan tellen, zou ik de (te zeldzame) uren met mijn zus Déanne en haar gezin en mijn broertje Anne Wieger niet willen missen. Alle andere familieleden, vrienden en bekenden die mij in de afgelopen jaren tot steun zijn geweest wil ik hierbij hartelijk bedanken. Speciaal wil ik René Vloon en Leo en Yvette van Brunschot noemen; zij hebben bewezen wat de waarde van echte vriendschap is. Paul Kuyt wil ik bedanken voor zijn begrip voor mijn "sorry, ik heb weer weinig gitaar gespeeld de afgelopen week".

De basis voor het werk, beschreven in dit proefschrift, is gelegd op het Pathologisch en Anatomisch Laboratorium van de Rijksuniversiteit Groningen. Daar werd ik als student begeleid door Prof. dr. J.W. Oosterhuis, een druk bezet patholoog-anatoom, met een alombekende wetenschappelijke en diagnostische kennis. Hij introduceerde mij op zijn karakteristieke wijze (twee rinkelende telefoons en een aantal wachtenden vóór en ná mij) in de intrigerende harmonie van normale weefsels, en de fascinerende verstoring ervan in maligniteiten. Met ondersteuning van Anke Dam, Janneke Buist, Frans Ramaekers en Hans Beck (Pathologie, Rijksuniversiteit Nijmegen) werden de eerste echte experimenten uitgevoerd, en werd vervolgens mijn eerste artikel over humane kiemceltumoren geschreven. In deze paar maanden hadden Prof. dr. J.W. Oosterhuis en ondergetekende niet veel, maar zeker wel efficiënt contact. Eerstgenoemde reikte mij mijn bul uit, en had geregeld dat ik enkele maanden kon gaan werken op de afdeling Anthropogenetica van de Rijksuniversiteit Leiden.

Vanuit mijn hoofdkwartier op "Nieuweroord" (de hunkerbunker voor ingewijden) stortte ik mij, onder de bezielende leiding van Peter Devilee, in het kloneren (voorheen voor mij een futuristisch begrip). In korte tijd had ik het idee onder vrienden te zijn. Het harde werken in combinatie met de onder andere talloze "borrels", etentjes, voelbalwedstrijden (terwijl ik helemaal niet zo van die sport hou), is voor mij het voorbeeld geweest hoe uniek het wereldje van wetenschappelijk onderzoek kan zijn. Margreethe, Surish, Cor, Peter, Ad, Bert T., Tim, Arn, Vincent, Lau, Johan, Bert B., Mette, Alfons, Cees, Jacqueline, en de (Katwijkse) portiers hebben deze periode voor mij onvergetelijk gemaakt.

Lang heb ik niet getwijfeld toen Prof. dr. J.W. Oosterhuis (voor mij toen al Wolter) mij vroeg om met hem een laboratorium, met financiële ondersteuning van de Nederlandse Kanker Bestrijding, op de Dr. Daniel den Hoed Kliniek in Rotterdam op te richten. Al spoedig werd Ad Gillis aan ons duo toegevoegd. Met ons drieën hebben wij de eerste kinderziekten van een nieuwe afdeling overleefd. De vlotte start is mede te danken aan de inzet van de verschillende diensten van de Dr. Daniel den Hoed Kliniek, zoals de technische-, economische/administrative-, civiele-, bouwkundige-, intern transport, personeel en organisatie, restaurant, bibliotheek en de afdelingen Pathologie, Moleculaire Biologie en Biochemie. In het bijzonder wil ik Lambert Dorssers bedanken. Ondanks zijn aversie tegen stropdassen heeft hij aan mij de door zijn vriendin gesmeerde boterhammen afgestaan, met mij zijn kamer gedeeld, en ons ondersteund waar mogelijk was. Onze

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Een aantal maanden heeft Mary Abraham, afkomstig van de groep van Prof. dr. G. Saunders (University of Houston, Texas), op ons laboratorium gewerkt. Dear Mary, it was a special experience to work and live with you. Your motivation to work is an example for most of us, and your ability to sleep "where-ever you lay your head" is amazing. I am happy that we met.

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Buiten onze afdeling zijn er ook vele anderen betrokken bij het onderzoek aan kiemceltumoren. Eens per twee maanden komen wij in Utrecht bij elkaar om de laatste stand van zaken te bespreken. De groep is te groot om iedereen persoonlijk te bedanken, maar ik heb de vergaderingen altijd als zeer stimulerend, en door de heterogeniteit van de onderwerpen als uitdagend (en vermoeiend) ervaren. Ik hoop dat ik nog lang deel mag uitmaken van deze inspirerende groep wetenschappers.

Onderzoek zoals beschreven in dit proefschrift is niet mogelijk zonder de ondersteuning van de specialisten uit de kliniek. Zij, in het bijzonder Prof.dr. H. Schraffordt Koops, Dr. D. Sleijfer (Academisch Ziekenhuis Groningen) en Dr. J.W. Hoeksta (Urologie, Den Bosch), wil ik hartelijk bedanken voor hun getoonde vertrouwen en medewerking.

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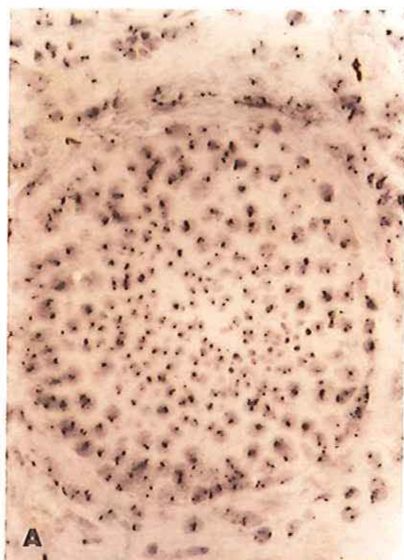
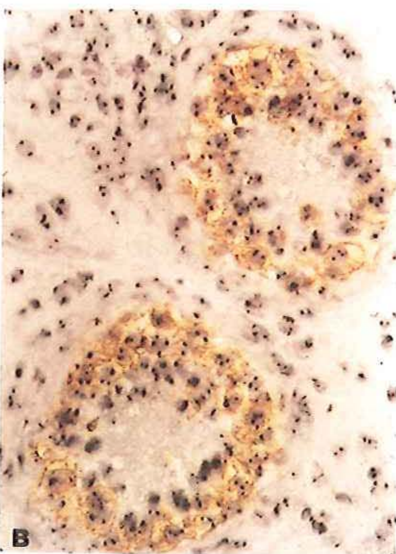
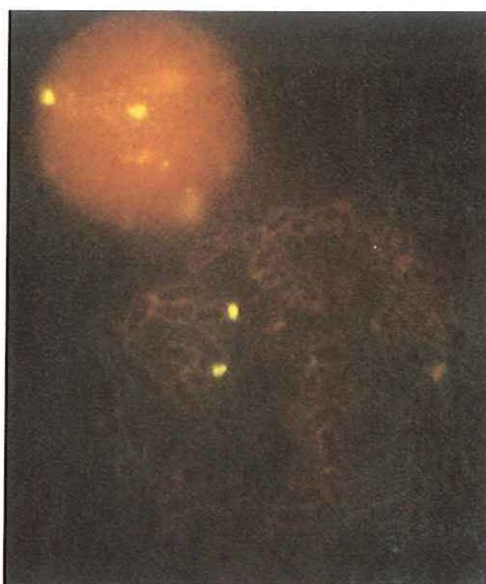
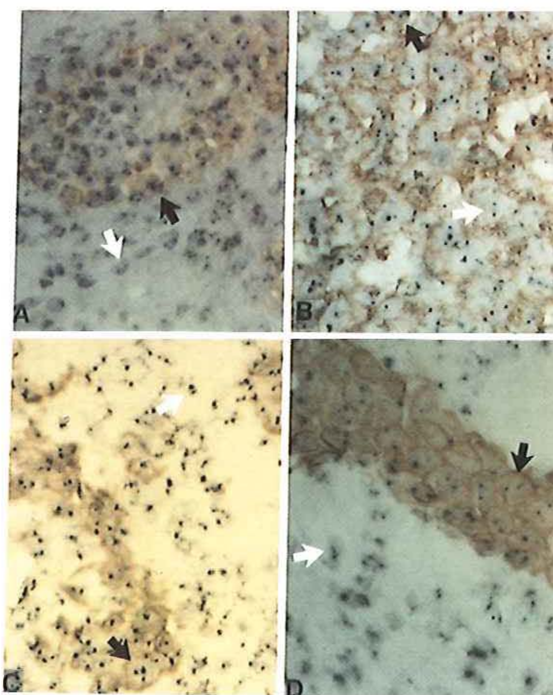




Chapter III. Figure 2.
Simultaneous immunohistochemical detection of placental like alkaline phosphatase expression and nonradioactive in situ hybridization with a chromosome 12 specific centromeric probe pα12H8 on a frozen 20 µm tissue section of case CIS-SE 3 (Table 2), illustrating a seminiferous tubule containing normal spermatogenesis (A) and CIS (B). Note numbers of signals/nucleus in the cells of spermatogenic lineage (1 or 2) and those in CIS (1, 2, 3 or 4).



Chapter II. Figure 2.
In situ hybridization of pα12H8 under high-stringency conditions (Devilee et al., 1988b) to a metaphase spread of a healthy donor. Arrows indicate resulting hybridization sites as detected by avidin-fluorescein isothiocyanate (FITC) treatment and propidium iodide counterstaining.



Chapter IV. Figure 1.
Representative illustrations of the numerical distribution analysis of centromeric regions of chromosomes 15 in different components of combined testicular germ cell tumors; (A) seminiferous tubules containing carcinoma in situ, (B) seminoma, both stained for placental like alkaline phosphatase, and (C) embryonal carcinoma, (D) teratoma, both stained for cytokeratins.

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