Leydig cell tumors of the testis: A molecular-cytogenetic study based on a large series of patients

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Abstract. The genetic features of the uncommon Leydig cell tumors (LCT) are largely unknown. Consequently, it is of great importance to elucidate the pathogenesis of testicular germ cell tumors by cytogenetic and molecular biological investigations. The purpose of the present study was the examination of cytogenetic features of these tumors in a large series of LCT. It comprised formalin-fixed, paraffin-embedded tissue samples from 25 LCT to analyze the chromosomal constitution using comparative genomic hybridization (CGH). In most of the studied cases, the aberrant cell population was additionally defined by interphase fluorescence *in situ* hybridization (I-FISH). Our molecular-cytogenetic study indicates chromosomal imbalances in the majority of our cases (21/25, 84%). The most frequent findings were gain of chromosome X, 19 or 19p and loss on chromosome 8 and 16.

Introduction

Leydig cell tumors (LCT) of the testis represent only 1-3% of testicular neoplasms. These tumors occur over a wide range of age, from childhood to senior adulthood. They are most common in the third to sixth decade, approximately one fourth of the reported cases occurred before puberty. LCT are always benign in children and in 90% of adults (1,2). The presenting features are painless testicular enlargement, gynecomastia (seen in 30% of adult patients and in 10% of children with virilization) and sexual dysfunction (3). Generally, the tumors are sporadic, although a familial occurrence in a father and his son has been reported (4). Occasionally, LCT are seen in patients with Klinefelter syndrome (5) and about 5-10% of patients have a history of cryptorchisms (6).

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Immunostaining for vimentin, inhibin, Melan A and calretinin is positive in over 90% of the cases and negative for PLAP (placental alkaline phosphatase) and cytokeratin (7,8).

The genetic features of these uncommon tumors are largely unknown. CGH is a suitable method to give an *in vivo* genomic overview of these uncommon tumors, which can achieve practical importance in diagnosis and prognosis.

DNA ploidy assessments and cellular proliferation augment established prognostic factors in predicting malignancy. Aneuploidy is more frequently seen in malignant than in benign LCT (8-11). Whereas the histomorphology of these tumors are well described, no reports are available in the literature of cytogenetic data. The objective of this study was to characterize the genetic alterations of a large LCT series and to correlate these results with clinicopathologic data.

Materials and methods

Tumor specimens and immunohistochemistry. Twenty-five cases coded as LCT of the testis from 1981 to 2004 were retrieved from the Institute of Pathology Innsbruck, Austria; Institute of Pathology Regensburg, Germany and the Department of Clinical Pathology Vienna, Austria.

Tissue sections were stained with haematoxylin and eosin, profiled immunohistochemically with antibodies against vimentin, cytokeratin, α-inhibin, Melan A, chromogranin, synaptophysin, AFP (α-fetoprotein), CD138, PLAP (placental alcaline phosphatase) and ß-HCG (beta human chorionic gonadotropin. Immunohistochemistry was performed using an automated immunostainer (Nexes, Ventana, Tucson, AZ, USA). The tumors are classified according to the WHO classification of testicular tumors (12). All 25 analyzed LCT were non-metastasizing tumors, which were diagnosed at a mean age of 42.2 years (range, 4-69 years). Two tumors in this study were diagnosed as malignant.

Comparative genomic hybridization (CGH). DNA from paraffin-embedded tumor material was extracted according to standard protocols. CGH technique that has been described previously in detail was applied (13). Briefly, tumor- and reference-DNA was labeled by nick translation with either biotin or digoxigenin (Roche Diagnostics, Mannheim, Germany). After co-precipitation with human Cot-1 DNA

Table I. Summary of clinical, histology and immunohistochemical data in 25 Leydig cell tumors of the testis.

No.	Localization	Vascular invation/ tumor behavior	Age	Immunohistochemical data
1	Testis r	No/benign	44	Positive for vimentin; negative for cytokeratin, chromogranin and synaptophysin
2	Testis r	No/benign	35	-
3	Testis l	No/benign	57	-
4	Testis l	No/benign	23	-
5	Testis	No/benign	38	-
6	Testis l	No/benign	50	-
7	Testis r	No/benign	38	-
8	Testis r	No/benign	64	Positive for inhibin; Ki-67 activity <5%
9	**Testis**	**Yes/malign tumor size >5 cm polymorph cells**	**55**	**-**
10	Testis	No/benign	40	-
11	Testis r	No/benign	23	-
12	Testis r	No/benign	29	Positive for vimentin, negative for cytokeratin; MIB-1 activity <0.5%
13	Testis l	No/benign	28	Positive for vimentin; negative for cytokeratin, PLAP and ß-HCG; MIB-1 activity 1-2%
14	Testis r	No/benign	62	Negative for AFP, ß-HCG and CD138 MIB-1 activity ~1%
15	Testis r	No/benign	36	Positive for vimentin, weak positive for Melan A; negative for PLAP and cytokeratin; MIB-1 activity <1%
16	Testis r	No/benign	4	-
17	Testis	No/benign	69	-
18	Testis l	No/benign	35	-
19	Testis	No/benign	60	-
20	Testis r	No/benign	24	-
21	Testis l	No/benign	36	Positive for inhibin, focal positive AFP and CAM 5.2 (<5%)
22	Testis l	No/benign	62	-
23	Testis l	No/benign	24	-
24	Testis l	No/benign	53	-
25	**Testis l**	**Yes/malign tumor size 4.8 cm 1/10 HPF**	**66**	**Positive for vimentin, calretinin and Melan A; negative for AFP, PLAP ß-HCG and chromogranin and synaptophysin MIB-1 activity ~5%**

Testis r, testis right; testis l, testis left; PLAP, placental alcaline phosphatase; AFP, α-fetoprotein; ß-HCG, human chorionic gonadotropin; HPF, high power fields; bold type, malignant cases.

hybridization was carried out in normal human metaphase cells for 3 days at 37˚C. For detection, the slides were stained with avidin-fluorescein isothiocyanate (Vector Labs Burlingame, CA) and anti-digoxigenin-rhodamine (Roche Diagnostics). Evaluation of the CGH preparations was performed using a fluorescence microscope (Zeiss Axioplan) equipped with a CCD camera (JAI M300) and the ISIS software (Metasystems, Altlussheim. Germany). Gains or losses were calculated as significant by the evaluation software with fluorescence ratio borderline values of 0.8 and 1.25, respectively. Pericentromeric, heterochromatic regions, telomeric regions and chromosome Y were excluded from the evaluation.

Interphase-fluorescence in situ hybridization (FISH). Parallel interphase-FISH was applied to define the involvement of copy number changes and for estimating the size of the aberrant cell population. Slide and probe preparation were performed according to the protocol of the manufacturer (Vysis Inc., Downers Grove, IL, USA). DNA probes for the centromeric region for chromosome X/Y, 2, 3, 8, 16 and 17, a locus specific probe for LSI 13q14 and a telomeric probe for chromosomes 19p were used.

Results

Twenty-five non-metastasizing tumor specimens from male patients were analyzed in this study. The histopathological,

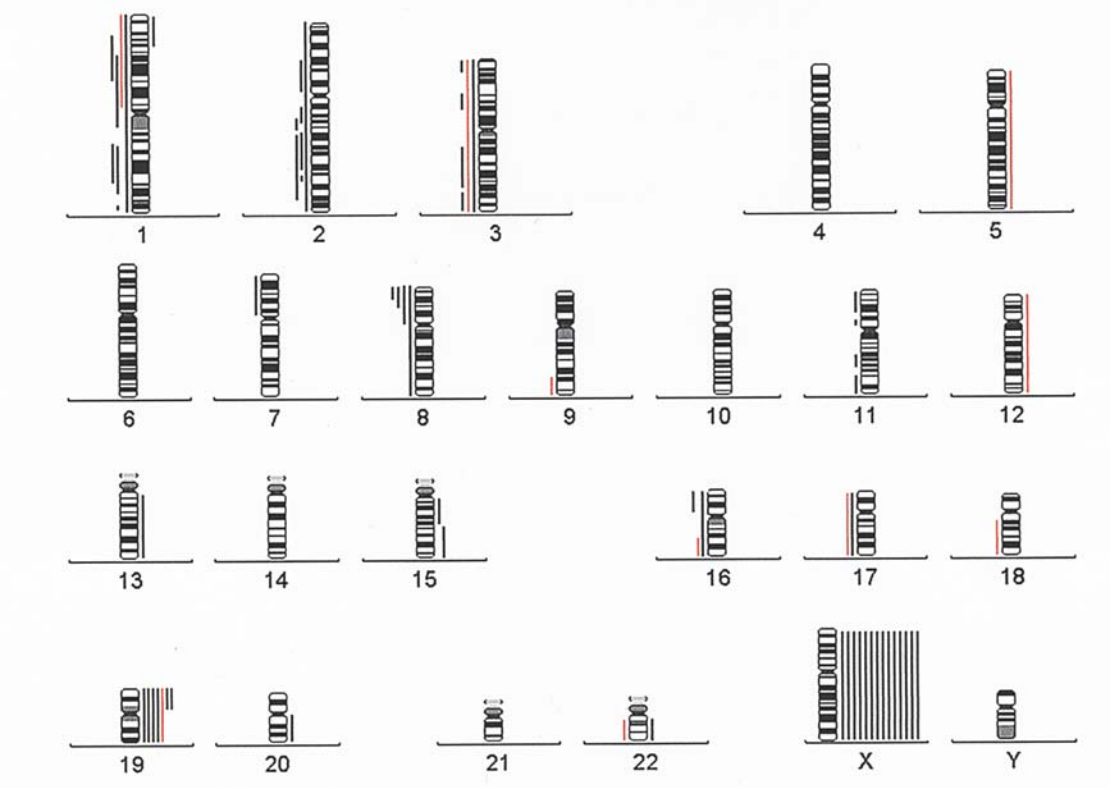

Figure 1. Summary of chromosomal imbalances detected by CGH in 21 Leydig cell tumors: vertical lines on the right side of the ideogram indicate gain, and those on the left loss of genetic material. Each line represents gain or loss in a single case; DNA copy number changes in our malignant tumors (case 9 is indicated as orange lines and case 25 as blue lines).

immunohistochemical and CGH findings were investigated.

The immunohistochemical analyses showed positive staining for vimentin and α-inhibin, and negative staining for cytokeratin, chromogranin, PLAP, AFP and ß-HCG. A summary of available clinical, histology and immunohistochemical data is given in Table I. The majority of the analyzed LCT showed chromosomal imbalances (21/25, 84%). The total number of DNA imbalances per tumor varied from case to case. Overall, 55 DNA imbalances were detected with a mean of 2.08 per case. Gains and losses were approximately equal with a gain/loss ratio of 1.26:1 (29 gains vs 26 losses).

A high genetic heterogeneity and involvement of chromosomes were found. Gain on chromosome X was detected at a high frequency (56%), followed by gain of chromosome 19 or 19p in 28% and losses on material of chromosome 1 (24%) and 8 or 8p in 16%. Chromosomal location of DNA gains and losses is given in Table I and Fig. 1.

Discussion

Genetic data on LCT in the literature are rare due the low incidence of these tumors. Only few mutation analyses in LCT are reported in the literature (14,15). Mutations of the luteinizing hormone receptor gene, located on 2p21 are known (16,17). To our knowledge, no cytogenetic data are available of LCT in the literature. This study is the first report on chromosomal aberrations in a series of LCT. The majority of DNA imbalances in our study were gains and losses of entire chromosomes, indicating missegregation of chromosomes as main mechanisms for producing aberrations in LCT.

Sex chromosomes, especially chromosome X seems to play an import role in oncogenesis of testicular germ cell tumors (18,19) and in LCT (20). The frequent finding of gain of chromosome X by CGH in the present study was confirmed by FISH, with mainly two copies of chromosome X, showing XXY/XX/XXYY or XXY/XXYY mosaicism (Table II). The distribution of the sex chromosomes in the present study suggests a heterogeneous population of near-diploid to near-tetraploid cells. The fact that in 56% of our cases of LCT showed gain on chromosome X is of interest, because overexpression of chromosome X was also a frequent observation in seminomas, non-seminomas and spermatocytic seminomas (21-23). The biological and clinical significance of numerical increase in X chromosomes in testicular germ cell tumors was suggested by enhanced expression of the 2 X-linked oncogenes *ARAF1* and *EKL1* (24).

Gain of chromosome 19 or 19p was observed as the second common aberration in this study, usually involving the entire chromosome (Fig. 1). Gain of chromosome 19 is reported in several other tumors (25,26), however, the gain of chromosome 19 should be interpreted with caution since chromosome 19 is prone to hybridization variability that may result in CGH artefacts. In three cases, we were able to carry out a successful FISH analysis with telomeric probes for 19p. In two of these cases the CGH result was confirmed by FISH.

Approximately 10% of LCT are malignant and metastases occur usually within 2 years after the initial tumor diagnosis (27,28). Malignant behavior in a prepubertal patient has not

Table II. Summary of CGH and I-FISH data in 25 Leydig cell tumors of the testis.

No.	CGH		FISH
	Losses	Gains	Losses and gains (% nuclei, copy number)
1	8pter-p21	X	Gain X (33)
2	None	X	Gain X (34)
3	None	19p, X	Gain X (40)
4	None	X	Gain X (33)
5	2[a]	19, X	Gain 19p (43, mainly 3-4 signals), gain X (31)
6	3[a]	13, 15q11.2-q21.1	Loss 3 (66, 0-1 copy), gain 13q14 (48, mainly 3-5 signals)
7	None	1pter-p33, 15q21.3-qter	Not done
8	None	X	Gain X (54)
9	**1p, 3, 9q32-qter**	**5, 12, 19**	**Loss 3 (60, 0-1 copy)**
	16q21-qter, 17		**Loss 17 (53, 0-1 copy)**
	18q, 22		**Gain 19p (32, mainly 3-4 signals)**
10	8p	None	Loss 8p22 (66, 0-1 copy)
11	None	None	
12	None	None	
13	1p31.3-q13.3; 1q23-q42.1	19, X	Gain X (24); gain 19 not confirmed
14	None	None	
15	None	X	Gain X (20)
16	None	19, 20q, 22q, X	Gain X (18)
17	1, 3, 7p, 8,11[a]	19p, X	Loss CEP 8 (64, 1 copy), 8q24 (55, 1 copy); gain X (43)
18	8pter-p22	19, X	Gain X (16)
19	1p34.2-p22.1, 1q23-q32.2	None	Not done
20	2q[a]	X	Gain X (35)
21	16, 17	None	Loss of 16 (79, 1 copy), loss of 17 (76, 1 copy)
22	2	None	Loss 2 (33, 1 copy)
23	None	X	Gain X (35)
24	None	None	
25	16p	X	Gain X (26)

[a]Imbalance with a clear shift, but the CGH profile shows only a partially significant imbalance (partially reaching the threshold); bold type, malignant cases.

been reported (6). The features indicating malignancy are presence of tumor size >5 cm, excessive mitotic rates (with 3-5 mitotic figures/10 HPF), tumor necrosis, vascular invasion, anaploid DNA value, cytologic atypia, increased MIB-1 activity (>5%) and a positive staining for p53 protein. Tumors that lack all these features are extremely unlikely to metastasize.

DNA ploidy status is reported as a useful prognostic indicator in these tumors (8,10,11). Several studies suggest, that the majority of benign LCT are diploid and the malignant tumors aneuploid. Our malignant tumor (case 9, from a 55-year-old patient) had a size >5 cm, vascular invasion and polymorphic cells. In this tumor the highest number of DNA alterations was found, including loss of 1p, 3, 9q32-qter, 16q21-qter, 17, 18q and 22, gains of entire chromosome 5, 12 and 19. Based on a tumor size of 4.8 cm, vascular invasion, mitotic figures 1/10 HPF and a proliferation index MIB-1 of 5%, case 25 was diagnosed as malignant tumor. Loss of 16p material and gain of chromosome X only were detected by CGH.

The molecular cytogenetic data in this study represent a first contribution to the poorly explored entity of LCT. Regarding the heterogeneous pattern of chromosomal aberrations, the significance of genetic lesions remains unclear. In conclusion, our data focus attention on the relevance of gain of chromosome X in the oncogenesis of these tumors that may be the basis for further examinations.

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