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The Leydig cell tumor Scaled Score (LeSS) A method to distinguish benign from malignant cases, with additional correlation with MDM2 and CDK4 amplification

Running Title: **Scaled score (LeSS) for predicting metastatic LCT**

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ABSTRACT

Aims: The aim was to investigate the morphological and molecular characteristics of Leydig cell tumors of the testis (LCT) for the identification of cases that may metastasize.

Methods and Results : Six parameters for a predictive model of the metastatic risk were evaluated in 37 benign and 14 malignant in LCTs of the testis (LCT scaled score = LeSS). The tumor size (benign LCTs: mean 1.33 cm, malignant cases: mean 4.4 cm) ($P < 0.001$) and other five parameters (infiltrative margins, necrosis, vascular invasion, mitotic count and nuclear atypia) showed significant differences (Wilcoxon's test $P < 0.001$). Eight metastatic tumors and one benign LCT had infiltrative margins. Foci of coagulative necrosis occurred in ten metastatic cases, while vascular invasion was identified in 9/14 metastatic cases and 0/37 benign LCTs. Benign LCTs showed < 2 mitoses/10 hpf, while high mitotic count (range 3 - 50 mitoses/10 HPFs) was a feature of malignant cases. These parameters were selected by an inferential analysis based on univariate logistic regression models to develop a score. A LeSS < 4 accurately identified all histologically and clinically benign LCTs. A LeSS ≥ 4 correctly identified all malignant LCTs. MDM2 and CDK4

immunostains were performed in all 51 cases: benign LCTs were negative, 3/11 malignant LCTs (27%) showed strong and diffuse immunopositivity and high levels of *MDM2* and *CDK4* amplifications by FISH analysis and by next generation sequencing .

Conclusion : We provide a new tool, a scaled score (LeSS), for the prediction of the malignant behavior in LCTs.

Key Words: Leydig cell tumor, immunohistochemistry, NGS, FISH

Introduction

Leydig cell tumors (LCTs) are neoplasms of variable biologic aggressiveness that are responsible for 10% of early pseudopuberty in children. LCTs can occur at any age in the adult life, sometimes presenting with gynecomastia or Cushing syndrome.

Most Leydig tumors are benign, and surgical resection is curative. Less than 10% of LCTs have a malignant course (1). Currently, there are no validated methods for the identification of cases that may metastasize. Some studies have proposed factors predictive of prognosis: the tumor proliferative index, mitosis, nuclear atypia, atypical mitosis, infiltrative margins, necrosis, high cellularity, vascular invasion, extratesticular spread, capsular invasion and size (2,3). However, none of the above-mentioned methods have been validated in independent populations. A classic study by Kim *et al.* of 40 Leydig cell tumors published in 1985 (3) found that mitotic count > 3 mitosis/10 HPFs, size > 5 cm, extratesticular spread, nuclear atypia, necrosis and vascular invasion were most predictive of malignancy, defined as the development of metastases.

In this study, we compared the morphological and molecular features of 37 benign LCTs surgically resected at the Fondazione IRCCS Istituto Nazionale dei Tumori di Milano with 14 malignant cases referred for a consultation.

Materials and Methods

Fifty-one LCTs occurring between 2000 and 2019 were retrieved from the archive files in the Department of Pathology of Fondazione IRCCS Istituto Nazionale dei Tumori di Milano. The series included 37 patients with primary consecutive benign LCTs excised at this institution and 14 patients with LCTs with malignant behavior referred to the senior author (MC) for a consultation. Clinical data, including patient age, symptoms at

presentation, laboratory values, follow-up data regarding tumor location, treatment modalities and current patient and disease status, were available for 50 patients. The cases spanned a period of 20 years, with follow-up ranging from 4-219 months. The material transfer agreement (MTA) was obtained for consultation cases from the original institutions. A mean of three slides were evaluated for each malignant case; mitotic count was assessed by three expert pathologists (M.C., B.P., and M.F.) in a blinded fashion, and a representative slide was selected for immunostaining. Original tumor parameters, such as the size and the presence of invasion of the spermatic cord were retrieved from a survey of the original surgical report. Nuclear atypia was scored 0 to 2. Uniform shape and nuclei with no atypia were scored as 0, small tumor cell nuclei with minimal variation in size and shape were scored as 1, and tumoral cells with large nuclei and/or nuclei with varied size and shape and macronucleoli were scored as 2. The mitotic count was determined in 10 fields with x40 objective lens (HPF). Lymphatic and vascular invasion, coagulative necrosis, soft tissue paratesticular invasion and infiltrative margins were assessed by H&E staining.

Immunohistochemistry and FISH

According to the availability of the sections in consultation cases, a panel of immunostains was assessed, including inhibin, calretinin, Ki67, SF1, synaptophysin, melan-A, CD 99 and chromogranin. The antibodies used and their technical specifications are summarized in Table I. Furthermore, IHC expression for MDM2 and CDK4 was assessed. The detection of MDM2 involved heat –induced epitope retrieval performed in Dako PT Link with EDTA buffer (pH 9.0) for 30 minutes on 4µm thick formalin-fixed paraffin-embedded sections. For CDK4, antigen retrieval was obtained by treatment with citrate buffer (pH 6.0) for 15 minutes. IHC for both MDM2 and CDK4 was performed using the EnVision FLEX+ detection system on an Autostainer Link 48 Dako platform, and strong nuclear expression in at least 50% of the cells was considered positive. Concurrent positive and negative controls were stained.

The ZytoLight (ZytoVision GmbH, Fischkai 1, D-27572 Bremerhaven, Germany) SPEC MDM2/CEN 12 Dual Color Probe was used to assess the MDM2 (12q15) copy number status, while the Vysis (Abbott Molecular, Des Plaines IL, USA) DDIT3 Break Apart FISH Probe Kit was used to assess the Cdk4 (12q13.3 covered by Spectrum Green 663 Kb telomeric end of the Probe Kit) copy number status. FISH experiments were performed on FFPE samples using standard protocols. Fifty tumor nuclei were scored using a Leica DM 6000B (Wetzlar, Germany) microscope at 100X magnification with the appropriate fluorescence filters. Images were captured using Cytovision software (v. 7.0, Leica).

Next Generation Sequencing

Next Generation Sequencing (NGS) was performed after DNA extraction from whole-mount sections. Libraries were prepared using the OncoPrint™ Comprehensive Assay v3 (Thermo Fisher) to identify hotspot mutations and copy number variations in 161 cancer-related genes. Sequencing was accomplished on an Ion S5 sequencing machine. Variant calling was carried out with the Variant Caller v.4.4.3.3 plug-in, and the variants were further filtered using Ion Reporter software v.4.4 (Thermo Fisher). Criteria for NGS data acceptance and variant calling were: avoidance of synonym mutations; coverage >500 reads; quality Phred score <0.0001; allelic frequency >5%. All variants were cross-checked on the ClinVar website (<https://www.ncbi.nlm.nih.gov/clinvar/>) and called with pathogenic or uncertain or unknown significance accordingly.

Statistical analyses

Seven prognostic factors were considered for the study: size, atypia, mitosis, vascular invasion, necrosis, infiltrative borders, and soft tissue invasion. The comparison of continuous variables (size) was performed using the Wilcoxon test, whereas the comparisons of discrete variables (nuclear atypia and mitosis) and continuous variables (vascular invasion, necrosis, infiltrative borders, and soft tissue invasion) were performed using the Fisher exact test. A P value < 0.05 was considered significant.

Seven univariate logistic regression models were applied to define the significant covariates best able to predict the outcome by estimating the coefficient, the standard error and the odds ratio. The results of these analyses were used to calculate a Leydig cell tumor scaled score (LeSS) to predict tumor behavior.

Results

Clinical features

Table II summarizes the clinical features of the 50 patients. Patient ages ranged from 37 to 71 years (mean, 41 years). The mean age of patients with the 37 benign LCTs was 37 years, and that of patients with the 14 metastatic LCTs was 52 years. After surgery the patients, followed-up in our hospital (mean 55 months, median 48 months, range 12-132 months), had no relapse. Primary tumor resection was performed by orchifunicectomy in 28 patients, while in 23 patients with smaller tumors, testis-sparing surgery was performed following the intraoperative diagnosis. Among malignant cases eleven patients underwent retroperitoneal lymphadenectomies: 10 were at our institution and 1 was at another hospital sent in consultation to one of the authors (MC). All of the eleven cases showed at microscopical level lymphnodal metastases. Regarding the three remaining patients two died one year and two years after orchiectomies for generalized disease with metastatic spread to the bone and lung, one had generalized disease at follow-up 7 months after the surgery with lung and bone metastases. Two of the eleven patients with metastatic disease occurring in the lymph nodes were lost to follow-up. Metastases occurred 2 to 51 months after orchiectomy (mean 16 months). Regarding benign LCTs, a history of gynecomastia was reported in 4 patients, and testicular swelling was reported in 20 patients showing hypoechogenic nodules by ultrasonography. The tumors were unilateral in 49 cases and bilateral in one benign LCT. The primary lesion was on the right side in 25 tumors and on the left side in 26. Among the clinical variables analyzed, age at presentation displayed the highest correlation

with malignancy, with a mean age of 52 years for metastatic patients and 37 years for benign patients ($p < 0.001$).

Pathological features

The other features of the benign and metastatic malignant LCTs are compared in Table III. Most LCTs were 1 to 5 cm in size (range 0.5-8.0 cm), and there was a statistically significant difference between benign (mean 1.33 cm) and malignant (mean 4.4 cm) cases (Wilcoxon's test p value < 0.001). One benign LCT was > 3 cm in size, while one malignant case showed a maximum size < 2 cm. Paratesticular soft tissue invasion occurred in 5 (10%) cases. Microscopic tumor patterns varied from solid to sheet-like to solid tubular or nested. Eight metastatic tumors and one benign LCT had infiltrative margins (Figure 1), while the others had circumscribed "pushing" borders (p value < 0.001). The tumor cells presented polygonal, with eosinophilic cytoplasm and variable-sized nuclei. Twenty-nine of 37 benign LCTs had grade 0 atypia, and 8 had grade 1 atypia. Among the 14 metastatic cases, 6 had grade 1 atypia, and 8 had grade 2 (Figure 2a,b). Atypia, as described above, was significantly associated with malignant behavior (p value < 0.001). Extensive foci of coagulative tumor necrosis occurred as a feature of malignancy in 10 of 14 malignant LCTs (Figure 3), while only two benign LCTs showed microscopic foci of necrosis (p value < 0.001). Vascular invasion was identified in 9/14 metastatic cases and in 0/37 benign LCTs (p value < 0.001) (Figure 4). Mitotic activity $\geq 1/10$ HPFs was observed in 20 of 37 (54%) benign LCTs, and no case showed more than 2 mitotic figures. Conversely, a high mitotic count ranging from 3 to 50 mitoses in 10 HPFs was a feature of malignant LCTs. The significant difference (p value < 0.001) between the two groups suggests ≥ 3 mitoses per 10 high-power fields as a feature suggestive of malignancy. Lymph node metastases were morphologically similar to those of the primary tumor, with somewhat increased nuclear atypia.

The immunohistochemistry results reported in Table IV show inhibin- α (focal expression) and SF-1 positivity in all cases, while other markers are variably expressed. Immunostains for MDM2 and CDK4 were performed in all 51 cases; 3/11 malignant LCTs (27%) showed strong and diffuse immunopositivity for MDM2 (Figure 5) and CDK4 in all tumor cells both in primary and metastatic tumors, while only 3/37 benign LCTs showed MDM 2 positive immunostaining in less than 10% of tumor cells and no positivity for CDK4 .

LeSS

Size, necrosis, infiltrative margins, mitosis and vascular invasion were selected by an inferential analysis based on univariate logistic regression models to develop a Leydig cell tumor scaled score (LeSS). The risk factors considered and relative scores are reported in Table V and Supplementary data 1- 2 . A LeSS < 4 accurately identified all histologically and clinically benign LCTs. A LeSS \geq 4 correctly identified all LCTs that had a metastatic course (Table VI).

Molecular analyses

FISH analysis

FISH showed high levels of *MDM2* and *CDK4* amplifications in 3 of 10 malignant LCTs from nine patients. Typically amplified cases displayed multiple (>20) gene copies per cell often arranged in tight clusters. FISH performed on both primary and metastatic tumors in one patient showed identical patterns of *MDM2* and *CDK4* amplifications (Figure 6).

Next Generation Sequencing

Our NGS comprehensive panel revealed gene alterations admissible according to our criteria in all the 10 *malignant* samples, as described in the mutation plot (Figure 7). Amplification of *MDM2* was found in three cases as well as *CDK4* and *CDK2* in two cases. Pathogenic mutations in *CTNNB1* (S33P and S33F), *ERCC2* (R227G) and *PMS2* (K356=) were found in other two cases. Mutations with uncertain pathological

significance were detected in *TSC2* (R1127K) in the case with primary and metastatic sample, together with a *NOTCH2* variant of unknown significance. Another mutation of uncertain clinical significance in *KIT* (K513I) was found in a case with concomitant amplification of *MDM2* and *CDK2*. Single mutations of unknown significance were found in *FANCD2*, *BRCA1*, *PI3KR1*, *RBI*, *PTCH1*, *SMO*, *SLX4*, *ERBB3* and *JAK1*.

Discussion

In 1985, Kim and coauthors evaluated six key parameters of malignancy (>3 mitotic figures per 10 high-power fields, size > 5 cm, infiltrative borders, nuclear atypia, vascular invasion and necrosis) in five metastatic LCTs of adult males and observed that at least four were always present (3). In the current WHO Classification of Tumours of the Urinary System and Male Genital Organs, the presence of two or more of the above-mentioned features are considered predictive of malignant behavior (4). A few cases of LCTs with a long follow-up have been reported in the last forty years (2,3,5,6,7,8), and although other markers have been explored for prognosis, little is known about the pathogenesis of LCT and how these markers may relate to clinically determined risk factors. A few biomarkers, such as Ki 67 (2,9,10,11), ploidy (11,12), gain of chromosomes X, 9 and 19 p and loss of chromosomes 8 and 16 (13), have been studied, but the significance of these findings remains unclear. More recently, one of the co-authors (AN), who evaluated a comprehensive genomic profile of 19 sex cord stromal tumors, observed *MDM2* and *CDK4* amplifications in 5 of 10 malignant LCTs (14). Here, we evaluated the presence of *MDM2* and *CDK4* copy number alterations by FISH and NGS analysis and their expression by immunohistochemistry in 11 malignant LCTs. We found *MDM2* and *CDK4* amplification and immunopositivity in 3 patients (27.7%). These alterations are related to cell-cycle control and disclose the potential of treatment of at least a subset of metastatic Leydig tumor with

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specific CDK4 inhibitors. Recently, a high prevalence of *MDM2* amplifications has been reported in many tumors, including breast cancer (13%), bladder cancer (9%), and sarcoma (57%) (15,16). The most common cooccurring molecular alteration was *CDK4* amplification, which was reported in 70% of *MDM2*-amplified cases. In the testis, the series reported by Saiki (17) showed *MDM2* amplification in only 2% of testicular germ cell tumors. In this study, we observed *MDM2* and *CDK4* coamplification in almost one-third of malignant LCTs and, conversely, no immunohistochemical expression in 37/37 (100%) benign LCTs. The NGS analysis found point mutations in *CTNNB1*, related to the WNT-B-Catenin pathway, that do not lead to specific therapeutic options, but have been reported in Sertoli cell tumor (SCT) (4,18). These two cases could represent Leydig cell-like SCTs according the mutational analysis and, recently, Zhang (19) have reported that simultaneous deletion of *WT1* gene and overactivation of *CTNNB1* in Sertoli cells led to Leydig cell-like tumor development in mice. No study has observed this “reprogramming” in testis tumors of adults and the morphology and negative *WT1* immunostaining seem to support our diagnosis of LCT. Single variant in *PMS2* was found in one case; more interesting would be the two variants found in *KIT* and *TSC2*. Unfortunately, the two variants found in these two genes are still of uncertain pathogenic potential. These observations provide a new perspective for the study of LCTs, although the future validation of these molecular data is needed.

The three patients with *MDM2* and *CDK4* amplification had retroperitoneal metastases 12, 16 and 21 months after the orchifuniclectomy, while no visceral metastases occurred. The patients were alive after 2, 3 and 10 years of follow-up. All three tumors showed at least 4 parameters related to malignancy: mitoses >3 (3/3), atypia (3/3), necrosis (2/3), angioinvasion (2/3), and infiltrative borders (2/3).

Surprisingly, the tumor size for the three malignant cases was less than 5 cm (2.5, 3.0, and 3.0 cm), and only one case had extension into parahilar soft tissue. There are no studies about the risk factors evaluated by a

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formal, rigorous statistical analysis. Here, we proposed a model for predicting tumor malignancy based on the statistical analysis of five parameters (LeSS). One of the key parameters included in the LeSS is size: in the past, size was considered a discrete variable, and the cut-off for malignancy was set at 5 cm (3). In recent years, a decrease in tumor size at diagnosis has been observed and reported in the literature (20). This may be due to the implementation of imaging that allows an early diagnosis and more frequent testis-sparing surgery for lesions < 2 cm. In line with the most recent literature (21), the patients in our study had smaller tumors. Moreover, only one of our benign LCTs had a size > 3 cm. Based on these observations, a new cut-off is proposed at ≥ 3 cm size. Regarding mitotic activity, the historical cut-off reported for progression risk is 3 mitotic figures/10 HPFs (3). Our data confirm this value of mitotic activity as a strong predictor of malignancy. The extragonadal extension (EE) has been observed in five malignant cases, while benign LCTs never showed EE. Leydig nests are frequently seen in the adipose tissue of spermatic cord or in the hilar tissue and may represent a diagnostic pitfall. The other parameters included in the LeSS are necrosis, infiltrative margins and vascular invasion. None of the above-mentioned factors, taken singularly, are predictive of malignancy, as stated by Rove in a series of clinical stage I testicular stromal tumors (TSTs) (22). In their systematic review, Rove and co-authors have evaluated pathological risk factors (including tumor size > cm.5, increased number of mitosis per HPF, positive margins, rete testis invasion, lymphovascular invasion, cellular atypia, necrosis) in two hundred ninety-two stage I testicular stromal tumors from 47 publications. They have performed the log-rank analysis comparing 5-year occult metastatic disease survival (OMDS) in 34 cases of TSTs with metastases and found that only the cases with 2 or more pathologic risk factors have a significant worse survival. In our study the inferential analysis of the five parameters elaborated in the LeSS model, based on the findings of a microscopic evaluation with H&E alone, effectively enables us to identify low- and high-risk classes for

metastatic behavior, and can be used in any pathology department. If available, FISH or NGS for *MDM2* and *CDK4* amplification might validate LCTs with borderline morphology minimizing the intraobserver variability relying on the assessment of histological parameters.

In conclusion, in our study, based on one of the largest series of malignant LCTs available in the literature, we provide a new tool for the prediction of the malignant behavior of LCTs based on molecular alterations and a Leydig cell tumor scaled score (LeSS) in these tumors that might help clinicians correctly manage and make clinical decisions in the follow-up of these patients.

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Supply of clinical data: *A. Necchi*

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List of online Supporting information

File Supplementary data 1 of : Case material : evaluated covariates and LeSS in metastatic LCTs.

File Supplementary data 2 Data set and descriptive analysis of statistical assays and inferential analysis

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Legends

Figure 1

Malignant Leydig cell tumor: in the border of the tumor presence of infiltrative margins .

Figure 2 Evaluation of prominent nuclear atypia in LCT.

A grade 1 atypia : Although the tumor cells have larger nuclei accompanied by conspicuous nucleoli in comparison to normal Leydig cells the variation in the size and shape is slight.

B grade 2 atypia : case with prominent nuclear pleomorphism . The cells are of varied shape and size with macronucleoli.

Figure 3 Extensive foci of coagulative necrosis observed in ten malignant LCTs.

Figure 4 Vascular invasion in a metastatic LCT.

Figure 5

Malignant Leydig cell tumor: mdm2 occurs as positive immunostaining in the large majority of Leydig cells.

Figure 6 Same LCT reported in Figure 5: *MDM2* amplification in tumoral cells (FISH assay).

Figure 7 Dot plot of genomic alterations in 10 samples of LCTs from 9 patients (sample 1 and sample 6 are the testis and retroperitoneal metastasis in the same patient with LT).

Table I Immunohistochemical panel

Antibody	Clone	Dilution	Source
Inhibin	R1	1:50	Serotec
Calretinin	DAK-CALRET1	1:200	DAKO
Ki67	MIB-1	1:400	DAKO
SF1	N 1665	1:25	Thermofisher
Synaptophysin	DAK-SYNAP	1:200	DAKO
Melan A	A103	1:400	DAKO
CD 99	MIC2-12E7	1:100	DAKO
Chromogranin	DAK-A3	1:100	DAKO
MDM2	IF2	1:40	Calbiochem
CdK4	DCS-35	1:200	Santa Cruz Biothecnology

Table II Clinical features

	No of Leydig cell tumors (%)		
	All tumors	Benign LCTs	Metastatic LCTs
Total number of tumors	51	37	14
Age (median, range) y	41,5 (19-71)	37,8 (19-71)	51,4 (27-64)
Time frame	12/00-01/19	05/01-04/19	12/00-01/19
Side			
Left	26	19	7
Right	25	18	7
Gynecomastia	4	-	4
Institute of original report			
INT Milan	37	37	-
Other	14	-	14
Follow-up (median, range) m	110 (4-219)	126 (4-219)	68 (7-165)

Table III Comparison of features between benign and malignant Leydig tumors.

Prognostic factors	Benign LCT (37 cases)	Malignant cases (14 cases)	P-value
Age	37 (range 19-71)	52 (range 28-64)	<0.05
Average size	1.33 (range 0.50-3.50)	4.41 (range 1.8 to 8)	<0.001
Infiltrative margins	1/37	8/14	<0.001
Necrosis	2/37	10/14	<0.001
Vascular invasion	0/37	9/14	<0.001
Mitoses >3 /10 high power fields	0/37 (mean 0.6)	10/14 (mean 10.2)	<0.001
Nuclear atypia	8/37 (grade 1)	14/14 (6 grade 1; 8 grade 2)	<0.001
Paratesticular soft tissue invasion	0/37	5/14	<0.001

Table IV Immunohistochemical stains in benign and malignant LCTs.

Antibody	Benign	Malignant
Inhibin	37/37(focal)	14/14 (focal)
Calretinin	37/37 (focal)	10/14
Ki67	<1%	1-10%
SF1	37/37	14/14
Synaptophysin	30/37 (Focal)	Not performed
Melan A	30/37	12/14
CD99	25/37	Not performed
Chromogranin	Rare positivities	0/14
MDM2	0/37	3/14
Cdk4	0/37	3/14

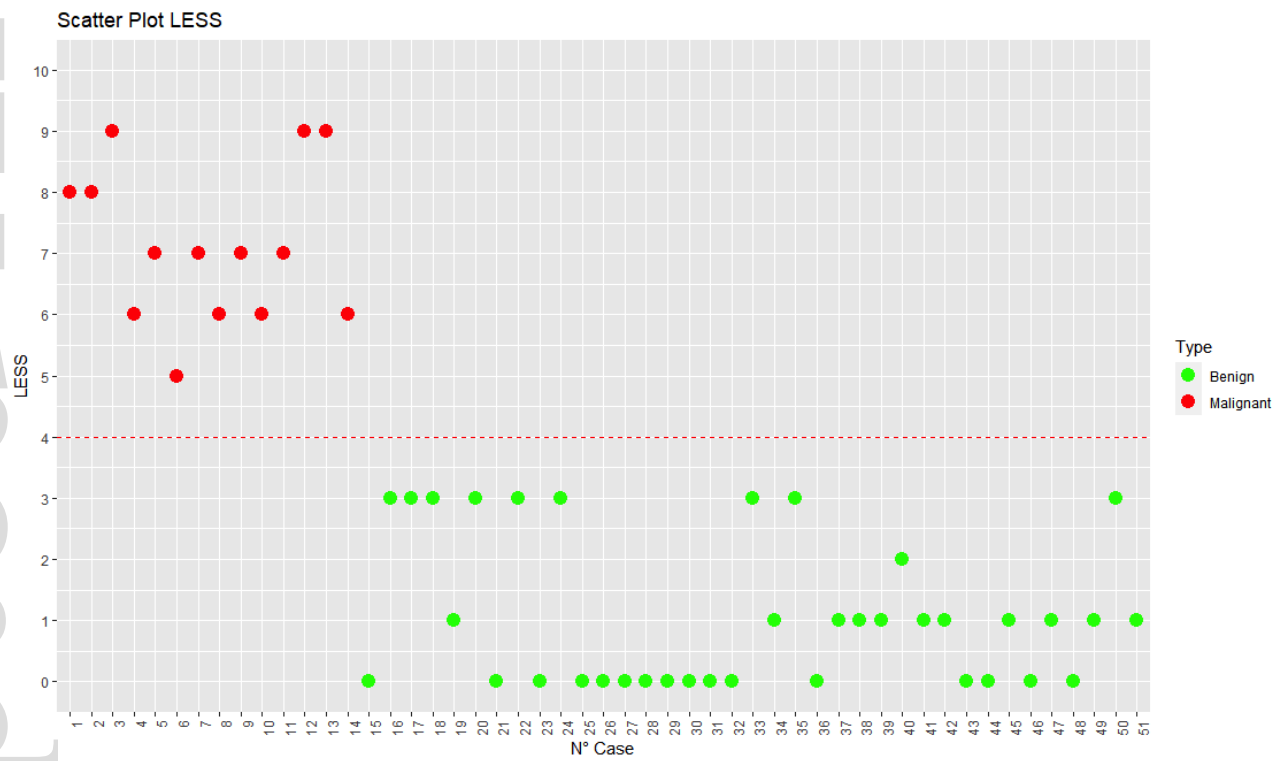
Table V

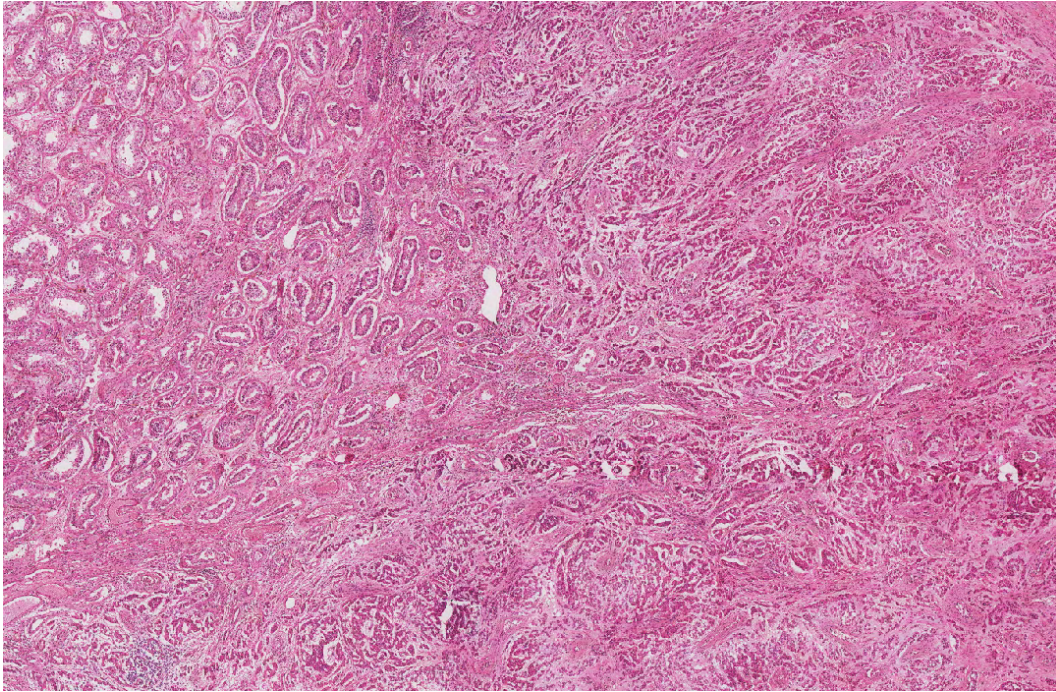
Leydig Scaled Score (LeSS): a score < 4 predicts clinically benign behavior.

Feature	Score (no.of points assigned)
Mitosis (x10 HPF) p	
0	0
1-3	1
≥ 4	2
Size	
cm. ≤ 1.5	0
cm. 1.5 to ≤ 2.5	1
cm > 2.5	2
Necrosis	
0	0
1	1
Infiltrative pattern	
0	0
1	1
Vascular Invasion	
0	0
1	1
Risk Class	Total
Low	0-3
High	4-7

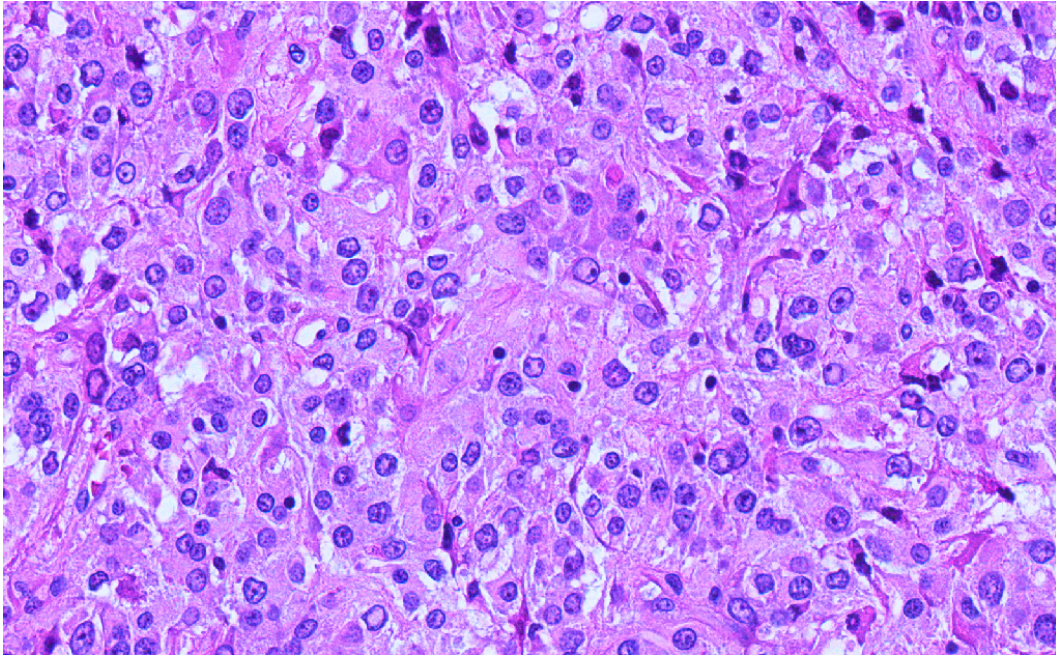
Table VI

Scattergram showing LeSS provided in 51 LCTs: a LeSS < 4 accurately identified all histologically and clinically benign LCTs (green dots); a LeSS ≥ 4 correctly identified all LCTs that had a metastatic course (red dots).

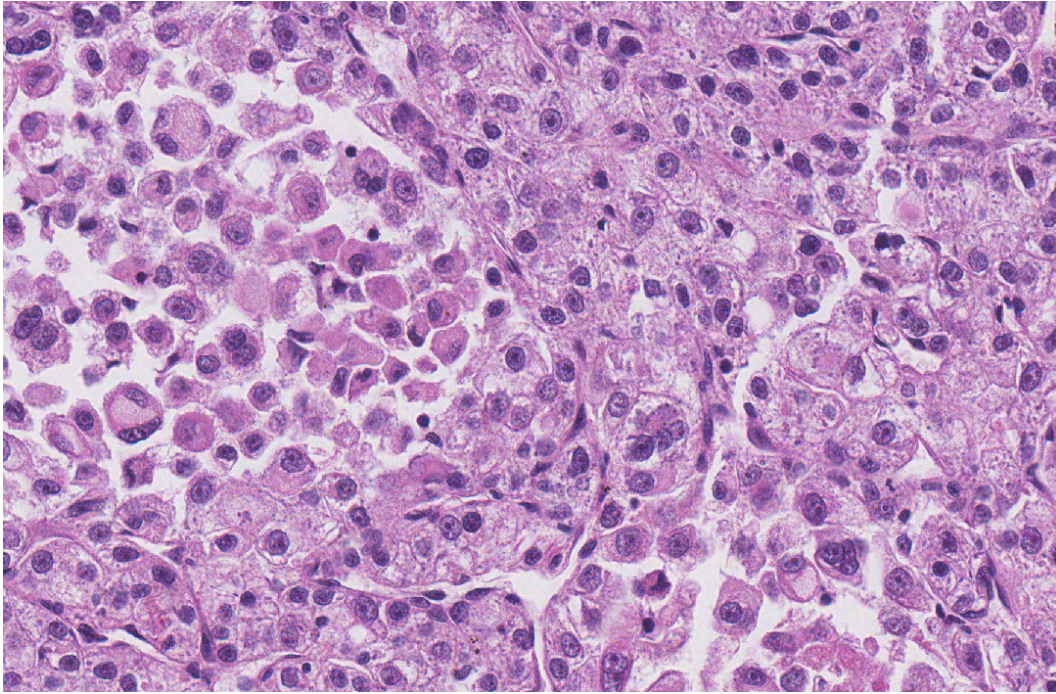




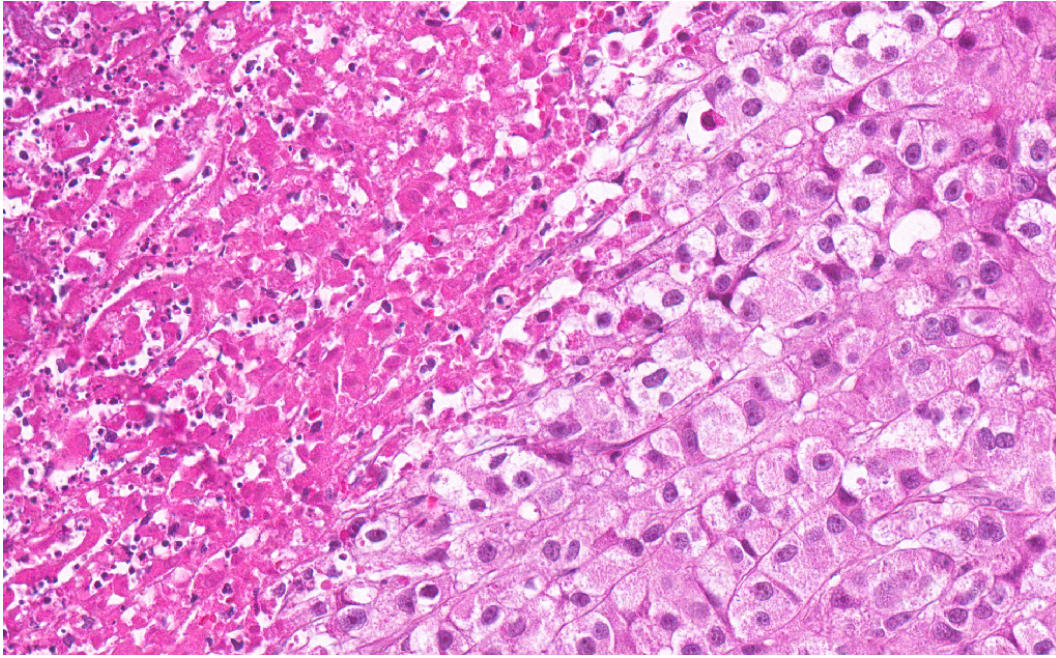
his_14225_f1.tif



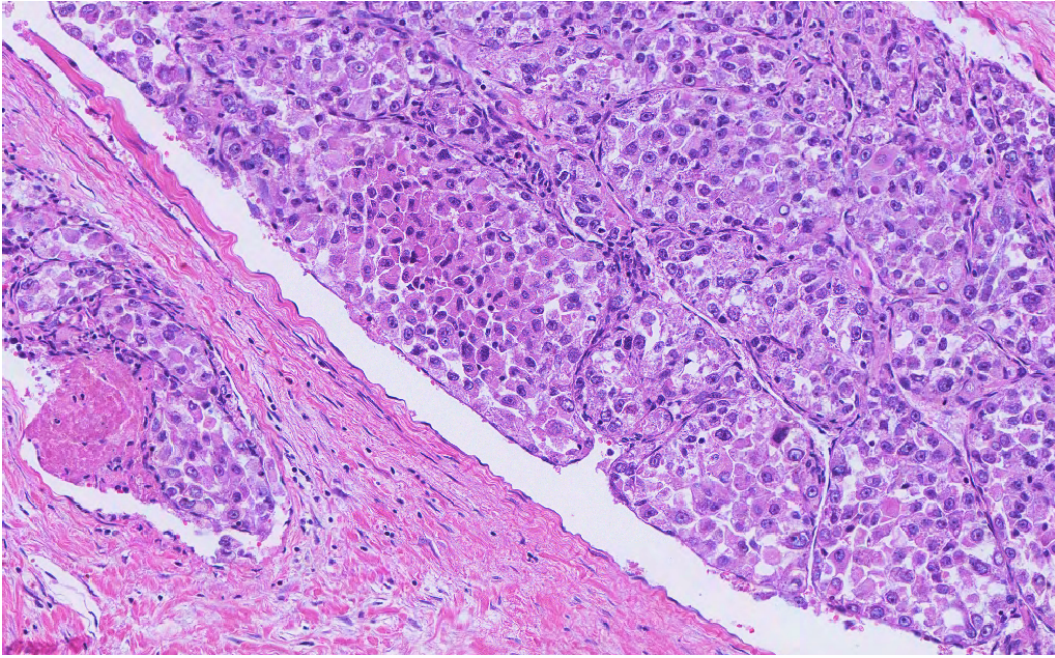
his_14225_f2a.tif



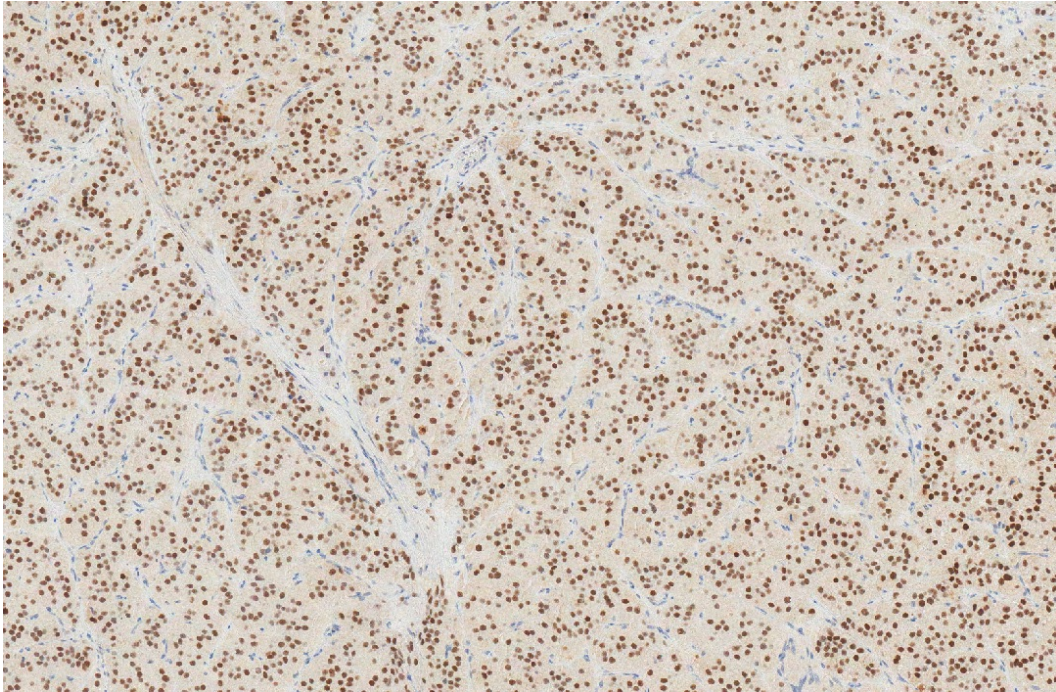
his_14225_f2b.tif



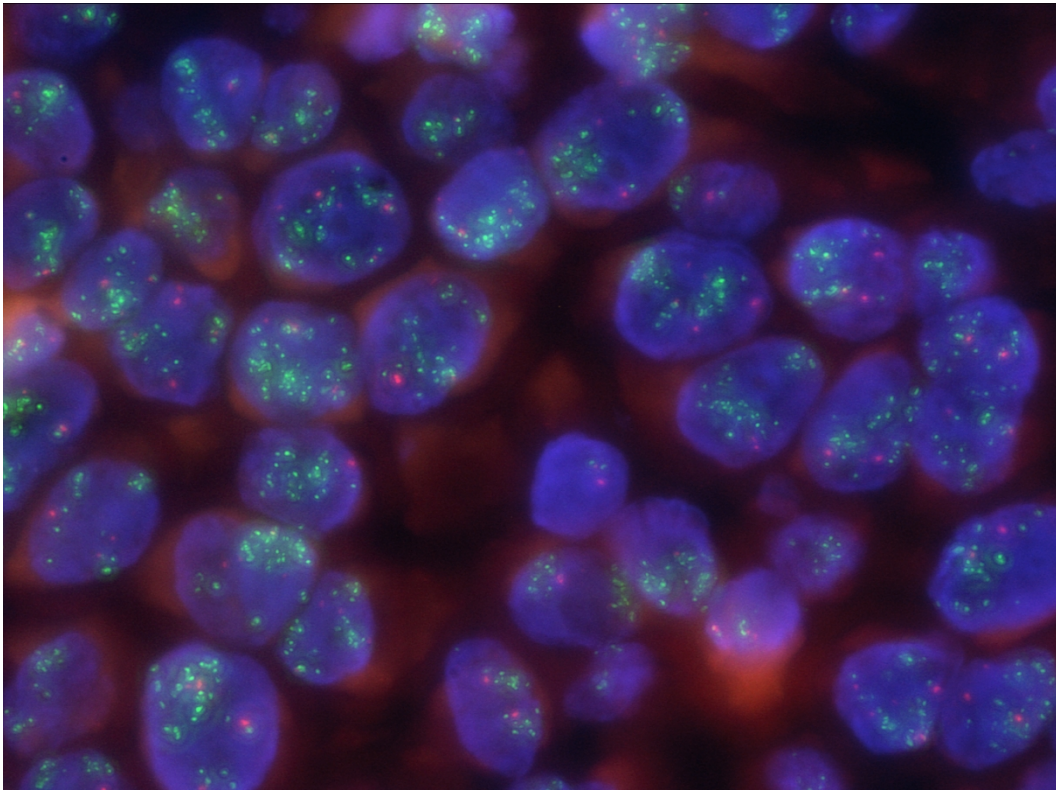
his_14225_f3.tif



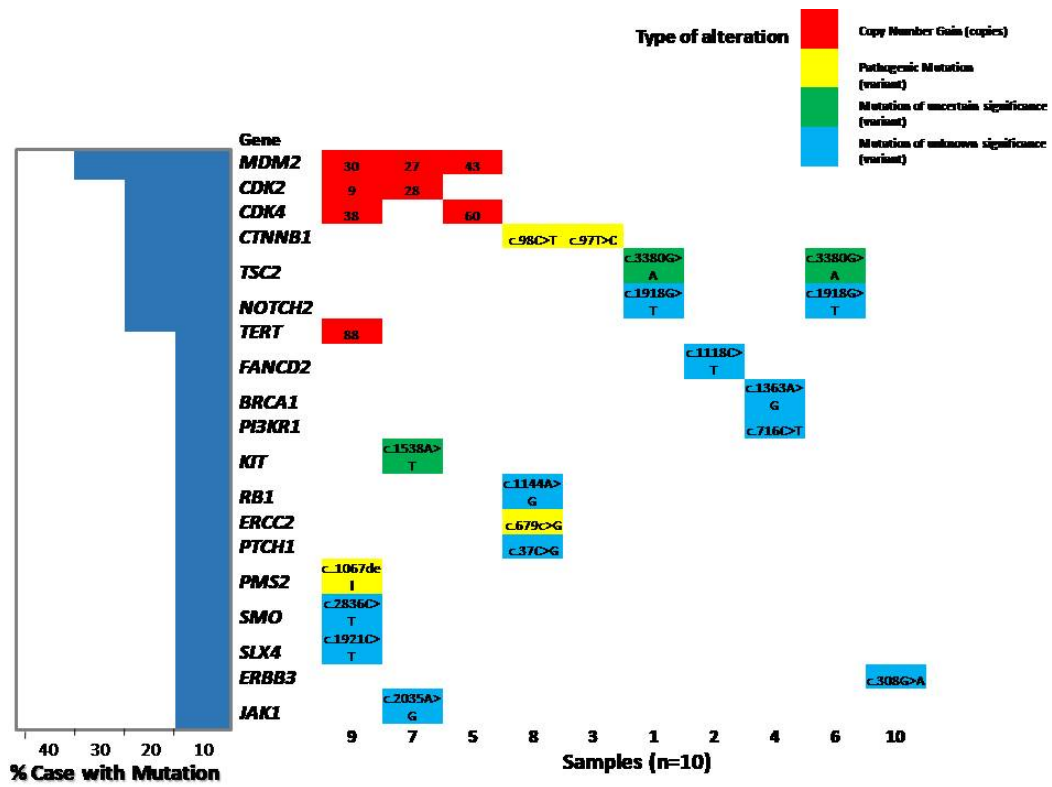
his_14225_f4.tiff



his_14225_f5.jpg



his_14225_f6.jpg



his_14225_f7.jpg