

Exploring chromosomal abnormalities and genetic changes in uterine smooth muscle tumors

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Smooth muscle tumors of the uterus are a diagnostically challenging group of tumors. Molecular surrogate markers reliably distinguishing between benign and malignant tumors are not available. Therefore, the diagnosis is based on morphologic criteria. The aim was to investigate a well-characterized group of challenging uterine smooth muscle tumors consisting of 20 leiomyomas, 13 leiomyomas with bizarre nuclei, and 14 leiomyosarcomas for copy number alterations, *MED12* mutations and *FH* deletions to search for potential diagnostically useful surrogate markers. *MED12* mutations were detected in 47, 15, and 25% of leiomyomas, leiomyomas with bizarre nuclei and leiomyosarcomas, respectively. *MED12* mutations in leiomyomas with bizarre nuclei were detected outside the hotspot region. *FH*-deletions were seen in 27, 30.8, and 25% of leiomyomas, leiomyomas with bizarre nuclei and leiomyosarcomas, respectively. By using copy number alteration profiling a clear separation of leiomyomas, leiomyomas with bizarre nuclei and leiomyosarcomas could not be observed. Copy number alterations revealed clear genetic similarities between leiomyomas with bizarre nuclei and leiomyosarcomas. Leiomyosarcomas showed a similar pattern of gains and losses as leiomyomas with bizarre nuclei, with additional copy number alterations and more homozygous losses and high-level amplifications compared to leiomyomas with bizarre nuclei. In conclusion, this study demonstrates that known *FH*-deletions, a recurrent molecular change in leiomyomas, occur in morphologically challenging variants of leiomyomas, leiomyomas with bizarre nuclei and leiomyosarcomas. Although *MED12* mutations are common in leiomyomas, they infrequently occur in leiomyomas with bizarre nuclei and leiomyosarcomas. The genetic similarities between leiomyomas with bizarre nuclei and leiomyosarcomas raise the intriguing possibility that uterine leiomyomas with bizarre nuclei and leiomyosarcomas are closely related and challenge the traditional concept that leiomyoma with bizarre nuclei is a tumor with just marked ‘degenerative’ cellular changes. These findings support the hypothesis that tumor progression within uterine smooth muscle tumors might occur.

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Introduction

Smooth muscle tumors are the most common mesenchymal tumors of the uterus. Approximately 70% of

women develop uterine leiomyomas and a quarter of them suffer from substantial clinical symptoms.¹ In contrast, though uterine leiomyosarcomas are rare, they are the most common sarcomas of the uterine body. Overall smooth muscle neoplasms are a heterogeneous, diagnostically challenging group of tumors with benign, malignant and intermediate tumor categories not infrequently causing diagnostic problems. Over the years a number of different morphologic tumor subtypes have been described. The benign uterine smooth muscle neoplasms include leiomyoma of usual type, cellular leiomyoma,² mitotically active leiomyoma,³ leiomyoma with bizarre nuclei (synonyms: ‘pleomorphic/symplastic’ leiomyoma and previously termed atypical

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leiomyoma according to the WHO 2003)^{4–7} leiomyoma with secondary hormonal changes,⁸ leiomyoma with hydropic degeneration,⁹ apoplectic leiomyoma¹⁰ and rare subtypes like plexiform leiomyoma,³ epithelioid leiomyoma,¹¹ myxoid leiomyoma¹² lipoleiomyoma,^{13,14} leiomyoma with hematopoietic elements,¹⁵ and neurilemmoma (schwannoma)-like leiomyoma.¹⁶ Leiomyomas mainly occur in women of reproductive age with a frequency as high as 77% if hysterectomy specimens are carefully investigated.³ In addition, there is strong evidence that these tumors are hormonally responsive and dependent.

From a molecular standpoint, leiomyomas are thought to represent separate clonal neoplasms arising independently from leiomyosarcoma within the uterus.¹⁷ Approximately half of leiomyomas are reported to have a normal karyotype on conventional cytogenetic analysis and the rest are characterized by a simple karyotype showing deletion of 7q, t(12;14), duplication of chromosome 12, and rearrangements involving 6p, 3q and 10q.¹⁸ The wide range of chromosomal alterations indicates that multiple different pathogenic mechanisms may be involved in tumor formation. Although in general, specific genetic patterns do not correlate with morphology, deletions of 1p have been reported to be associated with cellular and atypical morphology.¹⁹

Leiomyosarcomas account for < 2% of all uterine malignancies.^{20,21} They occur in the fifth and sixth decades of life presenting with symptoms like dysfunctional uterine bleeding and pelvic pain.^{22,23} Uterine leiomyosarcomas are aggressive tumors with a tendency to recur locally and metastasize mainly to the lung and liver. The 5-year survival rate varies between 45 and 60%.^{22–25}

Despite a number of molecular studies, the pathogenesis of uterine smooth muscle neoplasms needs further exploration, and reliable biomarkers to clearly separate diagnostically challenging cases are missing. Therefore, assessment of malignancy in uterine smooth muscle neoplasms is based on a landmark study by Bell *et al*³ using purely morphologic criteria. The crucial features include nuclear atypia, mitotic figures per 10 hpf, and the presence or absence of coagulative tumor cell necrosis.³ The diagnosis of malignancy is based on a combination of these criteria. Tumors short on these criteria are diagnosed as smooth muscle tumors of uncertain malignant potential.^{6,26,27}

Molecular data are available for uterine spindle cell leiomyosarcomas and leiomyosarcomas in soft tissues demonstrating variable cytogenetically complex numerical and structural changes of tumors, highlighting the degree of genomic instability.^{28,29} Frequent loss of heterozygosity particularly for chromosomes 10 and 13 has been described.^{30,31}

Recently, the first recurrent somatic mutations of the *Mediator Subcomplex 12* gene (*MED12*) have been demonstrated in benign and malignant uterine^{32,33} and extrauterine smooth muscle tumors³⁴ indicating that *MED12* mutations have a

crucial role in tumors with smooth muscle differentiation. In addition, *Fumarate Hydratase (FH)*-deletions and rearrangements of *high motility group protein 2 (HMGA2)* have been proposed as independent somatic driver mutations in leiomyoma.^{35–37}

The focus of this study was to explore copy number alterations, *MED12* mutational status, and *FH*-deletions in diagnostically challenging smooth muscle tumors with special emphasis on leiomyoma with bizarre nuclei, hormonally treated leiomyoma and uterine leiomyosarcoma.

Materials and Methods

Tumor Samples and Immunophenotyping

Twenty leiomyomas obtained from 15 patients including leiomyoma of usual type ($n=10$), hormonally treated leiomyoma ($n=6$), cellular leiomyoma ($n=1$), leiomyoma with very focal atypia (defined by very few isolated cells with degenerative nuclear atypia) ($n=2$), leiomyoma with prominent vascularity and edema ($n=1$), as well as leiomyoma with bizarre nuclei ($n=13$) obtained from 13 patients and leiomyosarcoma ($n=14$), including tissue from metastatic site, obtained from 8 patients (Tables 1, 2, and 3) were retrieved from the pathology archives of the Medical University of Graz, Austria, Yale University, USA and “Hospices Civils de Lyon”, Lyon, France. Four-micron hematoxylin and eosin stained sections generated from formalin-fixed paraffin embedded tissue were reviewed to confirm the diagnoses before inclusion in the study. Two pathologists (BLA, FM) independently reevaluated all cases for the following histologic features: type of margin (circumscribed or infiltrative), cell types (epithelioid, spindled, multinucleated), cytologic atypia (mild, moderate, severe), degenerative nuclear atypia, necrosis (absent, present, ischemic or coagulative), vascular invasion (absent, present), and mitotic rate (expressed as the number of mitotic figures per 10 high power fields (hpf) in the most mitotically active area, using a 40× objective and a 10× ocular, field size 0.25 mm²) (Tables 1, 2, and 3). Only unequivocal cases where both investigators came up with the identical diagnosis (leiomyoma, leiomyoma with bizarre nuclei, or leiomyosarcoma) were included in this study. No smooth muscle tumors with uncertain malignant potential were included in this study. We additionally performed immunohistochemical studies in all cases; the antibodies, clones, dilutions, pretreatment conditions, and sources are listed in Supplementary Table 4. We used the Envision Plus detection system (Dako, Carpinteria, CA, USA) for all antibodies. Appropriate positive and negative controls were included.

DNA Isolation

For DNA extraction, 10 μm sections were cut, tumor areas of interest were marked and manually

Table 1 Leiomyoma: overview of clinical data, morphology, and genetics

Patient No.	Tumor samples	No. of available tumors	Age	Size (cm)	Clinical history	Procedure	Diagnosis	Mitoses/10 hpf	Necrosis	MED12 mutation	FH deletion
1	Leiomyoma 1	1	45	12	Fibroid	Hysterectomy	Leiomyoma	0-1	Absent	Negative	FHdel
2	Leiomyoma 2	1	67	2.2	Bleeding	Hysterectomy	Leiomyoma	0-1	Absent	Negative	FHdel
3	Leiomyoma 3_1	2 samples from 2 different tumors	41	0.8	Fibroid	Hysterectomy	Leiomyoma	0	Absent	c.131G>T, p.G44V	
4	Leiomyoma 3_2 Leiomyoma 4_1		41 39	0.6 8	Fibroid Fibroid	Hysterectomy Hysterectomy	Leiomyoma Leiomyoma	0 0	Absent Absent	c.131G>A, p.G44D Negative	
5	Leiomyoma 4_2 Leiomyoma 5_1	2 samples from 2 different tumors	39 41	6 0.7	Fibroid Fibroid	Hysterectomy Hysterectomy	Leiomyoma Leiomyoma	0 0	Absent Absent	Negative c.131G>A, p.G44D	
6	Leiomyoma 5_1 Leiomyoma 6		41 32	1 3	Fibroid Uterine mass	Hysterectomy Hysterectomy	Leiomyoma Leiomyoma	0 0	Absent Absent	c.131G>A, p.G44D Negative	
7a	Leiomyoma 7_2	individual tumor	42	2	Menorrhagia	Hysterectomy	Leiomyoma	0-1	Absent	Negative	FHdel
8	Leiomyoma 8	1	52	Not known	Enlarging pelvic mass	Hysterectomy	Leiomyoma with edema and prominent vasculature	0-1	Absent	Negative	FHdel
9	Leiomyoma 9	1	37	14.3	Lupron treatment	Myomectomy	Leiomyoma	0-1	Present	c.100-10del10	
10	Leiomyoma 10	1	37	0.3	Lupron treatment	Myomectomy	Leiomyoma	0-1	Absent	c.100-64G>A	
11	Leiomyoma 11_1	2 samples from the same tumor	41	3.8	Lupron treatment	Myomectomy	Leiomyoma	0-2	Present	c.131G>A, p.G44D	
12	Leiomyoma 11_2 Leiomyoma 12		41 40	3.8 10	Lupron treatment Parasitic fibroid, suprapubic mass Lupron treatment	Myomectomy Myomectomy	Leiomyoma Leiomyoma	2 0-1	Present Present	c.131G>A, p.G44D c.131G>A, p.G44D	
13	Leiomyoma 13	1	32	12	Lupron treatment	Myomectomy	Leiomyoma	0-1	Present	c.130G>A, p.G44S	
14	Leiomyoma 14	1	51	Not known	Bleeding	Hysterectomy	Cellular leiomyoma	0-1	Absent	Negative	
15	Leiomyoma 15	1	61	2.2	Benign ovarian mass	Hysterectomy	Leiomyoma with focal atypia	0-1	Absent	c.107 T>G, p.L36R	
7b	Leiomyoma 7_1	Individual tumor	42	0.5	Menorrhagia	Hysterectomy	Leiomyoma with focal atypia, bilateral ovarian teratoma	0-1	Absent	Negative	

Table 2 Leiomyoma with bizzare nuclei: overview of clinical data, morphology, and genetics

Patient no.	Tumor sample	No. of available tumors	Age	Size (cm)	Clinical history	Procedure	Diagnosis	Mitoses/10 hpf	Necrosis	MED12 mutation	FH deletion	Follow up
1	Leiomyoma with bizzare nuclei 1	1	49	10	Rapidly growing tumor	Hysterectomy	Leiomyoma with bizzare nuclei	2	Present	Negative		NA
2	Leiomyoma with bizzare nuclei 2	1	36	20	Pelvic mass	Hysterectomy	Leiomyoma with bizzare nuclei	1-2	Absent	Negative	FHdel	NA
3	Leiomyoma with bizzare nuclei 3	1	30	8.5	Fibroid	Myomectomy	Leiomyoma with bizzare nuclei	0-1	Absent	Negative	FHdel	NA
4	Leiomyoma with bizzare nuclei 4	1	40	7	Fibroid	Hysterectomy	Leiomyoma with bizzare nuclei	2	Absent	Negative	FHdel	66 mo
5	Leiomyoma with bizzare nuclei 5	1	45	2.5	Fibroid	Hysterectomy	Leiomyoma with bizzare nuclei	4	Absent	Negative		94 mo
6	Leiomyoma with bizzare nuclei 6	1	67	3	Bleeding	Hysterectomy	Leiomyoma with bizzare nuclei	0-1	Absent	Negative		14 mo
7	Leiomyoma with bizzare nuclei 7	1	50	7	Fibroid	Hysterectomy	Leiomyoma with bizzare nuclei	2	Absent	Negative		74 mo
8	Leiomyoma with bizzare nuclei 8	1	74	7	Fibroid	Hysterectomy	Leiomyoma with bizzare nuclei	0-1	Absent	NA		60 mo
9	Leiomyoma with bizzare nuclei 9	1	73	2.5	Fibroid	Hysterectomy	Leiomyoma with bizzare nuclei	2	Absent	c.122T>C, p.V41A		50 mo
10	Leiomyoma with bizzare nuclei 10	1	36	5.5	Fibroid	Hysterectomy	Leiomyoma with bizzare nuclei	2	Absent	c.156T>C, p.(=)		105 mo
11	Leiomyoma with bizzare nuclei 11	1	50	5	Fibroid	Hysterectomy	Leiomyoma with bizzare nuclei	5	Absent	NA		NA
12	Leiomyoma with bizzare nuclei 12	1	38	Not known	Fibroid	Myomectomy	Leiomyoma with bizzare nuclei	3	Absent	NA		NA
13	Leiomyoma with bizzare nuclei 13	1	32	4	Uterine mass	Hysterectomy	Leiomyoma with bizzare nuclei	4	Absent	Negative	FHdel	NA

Abbreviations: mo, month free of disease; NA, not available.

Table 3 Leiomyosarcoma: overview of clinical data, morphology, and genetics

Patient no.	Tumor sample	No. of available tumors	Age	Size (cm)	Clinical history	Procedure	Diagnosis	Mitoses/10 hpf	Necrosis	MED12 mutation	FH deletion	Follow up
1	Leiomyosarcoma 1_1	3 sample from 3 tumors	51	Not known	Primary, enlarged uterus	Hysterectomy	Leiomyosarcoma (G3)	43	Present	c.131G>A, p.G44D Negative		NA
	Leiomyosarcoma 1_2		51	Not known	Metastasis after 4a	Metastasectomy	Leiomyosarcoma metastasis	35	Present			
	Leiomyosarcoma 1_3		51	Not known	Metastasis after 7a	Metastasectomy	Leiomyosarcoma metastasis omentum	39	Present		Negative	
2	Leiomyosarcoma 2	1	80	8	Primary uterus, enlarged uterus, LMS with osteoclastic giant cells	Hysterectomy	Leiomyosarcoma with osteoclastic giant cells (G3)	18	Present	NA		NA
3	Leiomyosarcoma 3	1	59	8	Primary uterus, postmenopausal bleeding	Hysterectomy	Leiomyosarcoma with epitheloid features and clear cell changes (G3)	35	Present	Negative		NA
4	Leiomyosarcoma 4	1	57	10	Primary uterus	Hysterectomy	Leiomyosarcoma (G2) and incidental endometrioid carcinoma	2	Present	c.131G>C, p.G44A het		DD
5	Leiomyosarcoma 5	1	46	5	Primary uterus	Hysterectomy	Leiomyosarcoma (G3)	32	Absent	Negative		DD
6	Leiomyosarcoma 6	1	51	9	Primary uterus	Hysterectomy	Leiomyosarcoma (G3)	12	Present	NA		DD
7	Leiomyosarcoma 7_1	2 sample from the same tumor	61	10	Primary uterus	Hysterectomy	Leiomyosarcoma (G3)	19	Present	Negative	NA	DD
	Leiomyosarcoma 7_2		61	10		Hysterectomy	Leiomyosarcoma (G3)	27	Present	Negative	FHdel	
8	Leiomyosarcoma 8_1	4 samples from the same tumor	58	14	Primary uterus	Hysterectomy	Leiomyosarcoma (G3)	22	Present	c.204+58G>C het	FHdel	DD
	Leiomyosarcoma 8_2		58	14		Hysterectomy	Leiomyosarcoma (G3)	25	Present	c.204+58G>C het	FHdel	
	Leiomyosarcoma 8_3		58	14		Hysterectomy	Leiomyosarcoma (G3)	18	Present	c.204+58G>C het	FHdel	
	Leiomyosarcoma 8_4		58	14		Hysterectomy	Leiomyosarcoma (G3)	17	Present	c.204+58G>C het	FHdel	

Abbreviations: DD, died of disease; NA, not available.

needle-microdissected. DNA extraction was performed with a DNA isolation kit (Qiagen) according to the protocol 'Isolation of genomic DNA from formalin-fixed paraffin embedded tissue sections'. The quality of the DNA was evaluated using a multiplex PCR approach.^{38,39} Samples with three or four bands on a 1.5% agarose gel were used for array-CGH analysis.

Array CGH

Array CGH detects copy number changes in a test DNA compared to a reference DNA at a high resolution. For this study 60k arrays from Agilent Technologies were used. Samples were labeled with the Bioprime Array-CGH Genomic Labeling System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 500 ng test DNA and reference DNA (Promega, Mannheim, Germany) was differentially labeled with dCTP-Cy5 or dCTP-Cy3 (GE Healthcare, Piscataway, NJ, USA). Slides were scanned using a microarray scanner (Agilent Technologies, Santa Clara, CA, USA). Data normalization and calculation of ratio values were conducted employing Feature Extraction software from Agilent Technologies. Data analysis was performed using the software Genomic Workbench 5.0.14 (statistical algorithm: ADM-2; alteration threshold: 8.0; moving average window: 2 MB; Fuzzy zero: off; consecutive clone filter: 10) from Agilent Technologies. Log ratios higher/lower 0.3 were considered as copy number change.

Mutational Analysis of *MED12*

MED12 exon 2 was amplified using Qiagen HotStart PCR MasterMix (Qiagen, Hilden, Germany) with primers MED12_E2_F: 5'-TTCTACACGGAACCCCTC CTC-3' and MED12_E2_R: 5'-GGCAAACCTCAGCCAC TTAGG-3') according to the manufacturer's recommendations. Briefly, we used 0.5 µl of DNA (corresponding to 20–50 ng), 1 µl of 10 µM primer in a final reaction volume of 11 µl. After diluting the PCR product 1:2, 1 µl was directly used for sequencing using Big Dye Terminator v3.1 Cycle Sequencing Kit in a final volume of 10 µl with 0.4 µl of 10 mM primers. Sequencing reactions were purified using Sephadex. Both DNA strands were sequenced on an ABI3130 Genetic Analyzer and mutations on one strand were always confirmed on the opposite strand. DNA sequence analysis was performed using SeqScape software (Applied Biosystems, Carlsbad, CA, USA). Sequences were aligned according to RefSeq entry NM_005120.

Statistics

To summarize copy number alterations of samples and to identify overlapping copy number alterations within each sample group, we subjected our data to

the Genomic Recurrent Event Viewer (GREVE) algorithm.⁴⁰ In order to identify recurrent changes, the tumor sample with the highest amount of copy number alterations was selected of those patients with two or more samples. Details on settings of the software parameters are available on request.

Comparison of the percentage of aberrant genomic regions for each subgroup was performed in R using the `wilcox.test` function.⁴¹ Log₂-ratios from the array-CGH raw data were clustered hierarchically by the `hclust` function of R using Manhattan distance in order to identify phylogenetic relationships between copy number profiles of leiomyomas, leiomyomas with bizarre nuclei, leiomyosarcomas, and tumor samples from the same patients in a distance matrix (from Manhattan distances).⁴¹

Results

Clinical Features

The clinical features of the 36 patients in our study are summarized in Tables 1, 2, and 3. The age at presentation ranged from 32 to 67 years (median age 44 years), from 30 to 74 years (median age 48 years), and from 46 to 80 years (median age 58 years) in the leiomyoma, leiomyoma with bizarre nuclei, and leiomyosarcoma groups, respectively. The tumor size ranged from 0.3 to 12 cm (median size 5 cm), from 2.5 to 20 cm (median size 7 cm), and from 5 to 14 cm (median size 9 cm) in the leiomyoma, leiomyoma with bizarre nuclei, and leiomyosarcoma groups, respectively. In the leiomyoma group, follow-up was only available for 5 patients none of them showed a recurrent tumor or development of a leiomyoma in another anatomic location with a follow-up time of at least 4 years. Follow-up for patients suffering from leiomyoma with bizarre nuclei was available in seven cases. No disease recurrence occurred with a follow-up between 14 and 105 month (median 66 month).

In the leiomyosarcoma group, follow-up was available in five patients all of them died of disease. One patient was lost in follow-up after metastasectomy and for two patients no follow-up was available.

Macroscopic Features

All leiomyomas were well circumscribed. These tumors showed a homogenous gray white color and a firm, rubbery consistency with the exception of hormonally treated leiomyoma, and one leiomyoma with bizarre nuclei. The leiomyomas with bizarre nuclei (Table 2: pt.1) revealed a more yellowish cut surface and a softer consistency. Hormonally treated leiomyomas additionally demonstrated areas of hemorrhage and infarction.

All leiomyosarcomas were less well circumscribed grossly. They were characterized by a softer consistency and on cut surface the color varied from

gray to cream. In the majority of leiomyosarcomas, necrosis appearing as yellow or green areas and red to brown areas of hemorrhage were present (Table 3).

Microscopic Features

Ten evaluated leiomyomas showed classic morphologic features. The tumors were composed of intersecting fascicles of spindle cells. The spindle cells showed bland elongated nuclei with blunt ends and eosinophilic cytoplasm. Nuclear atypia, necrosis, and mitotic activity were not present (Table 1: pts.1-7a and Figure 1a). One leiomyoma showed prominent vascularity and an edematous matrix (Table 1: pt.8).

The hormonally treated/infarcted leiomyomas (Table 1: pts.9-13) showed identical morphology to the leiomyomas of usual type; however, areas of ischemic necrosis were always seen. Ischemic-type

necrosis was defined as an area of necrosis surrounded by a zone of granulation tissue and hyalinized tissue typical for ischemia (Figure 1b). The cellular leiomyoma (Table 1: pt.14) showed classic morphologic features; however, the cellularity was significantly higher than that in usual leiomyomas. In two otherwise usual leiomyomas (Table 1: pts. 15, 7b), very focal cells with nuclear, degenerative atypia were seen, and one of these was associated with a bilateral ovarian teratoma (Table 1: pt.7b). Leiomyoma with very focal atypia was defined by only very few isolated tumor cells with degenerative-type atypia in an otherwise classic leiomyoma background (Figure 1c).

All 13 leiomyomas with bizarre nuclei were characterized by the presence of cells with enlarged and pleomorphic nuclei. In all cases, the bizarre nuclei were interspersed with bland smooth muscle cells. Multinucleated tumor cells were present

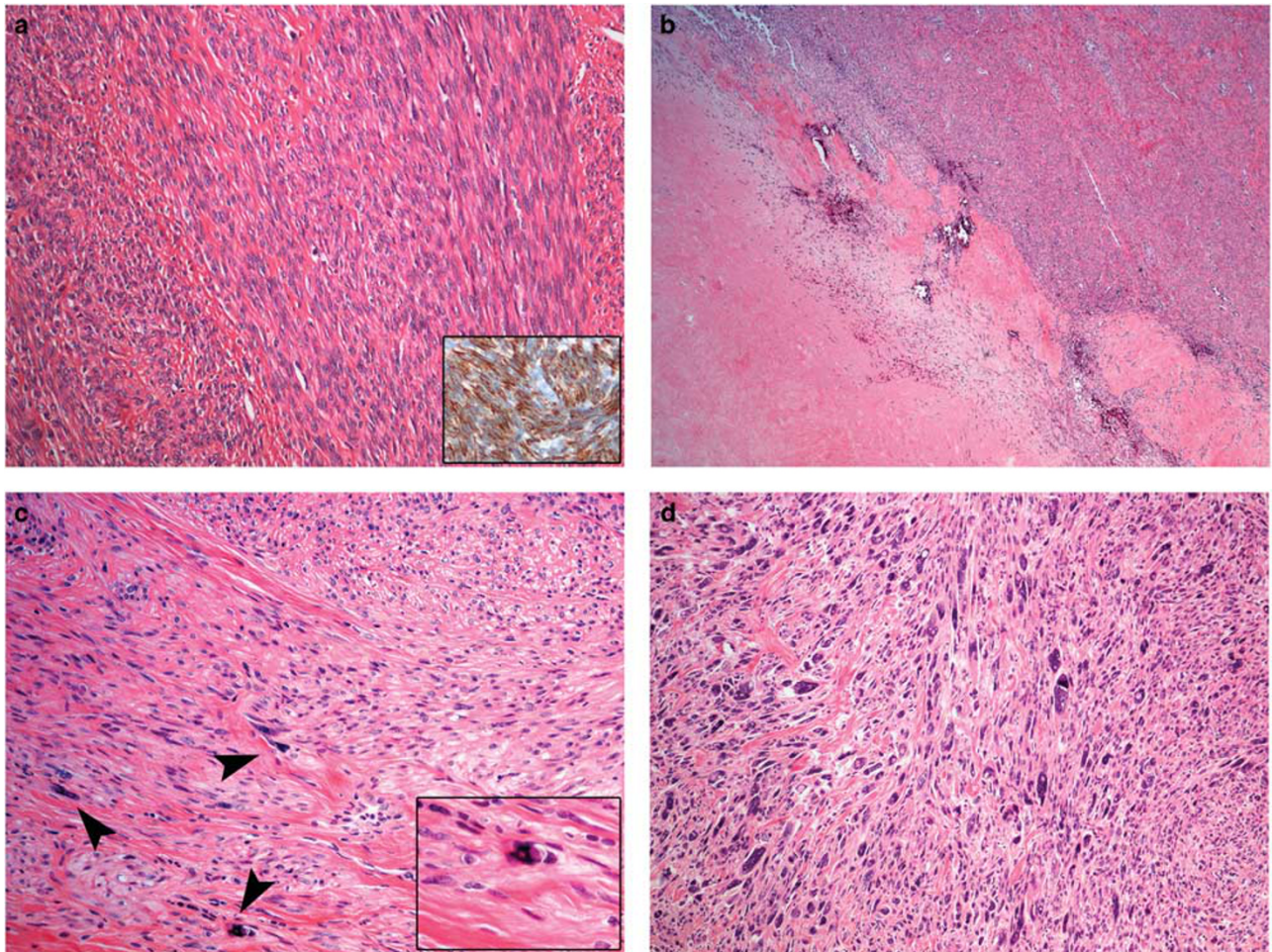


Figure 1 (a) Classic example of a leiomyoma with expression of Desmin (inset). (b) Leiomyoma after the Lupron treatment. Ischemic-type necrosis surrounded by a zone of granulation tissue and hyalinized tissue typical for ischemia. (c) Leiomyoma with very focal atypia showing isolated atypical cells within an otherwise classic leiomyoma. Cell with degenerative atypia (inset). (d) Leiomyoma with bizarre nuclei with enlarged and pleomorphic cells recognizable at low power examination. (e) Leiomyoma with bizarre nuclei: cells with enlarged nuclei with coarse chromatin, occasional pleomorphic nuclei with 'smudged' (degenerative type) chromatin pattern, nuclear pseudo inclusions, and abundant eosinophilic cytoplasm. (f) Leiomyosarcoma: cells with nuclear atypia and brisk mitotic activity. (g) Leiomyosarcoma: SMA expression by immunohistochemistry (h) Leiomyosarcoma: Desmin expression by immunohistochemistry.

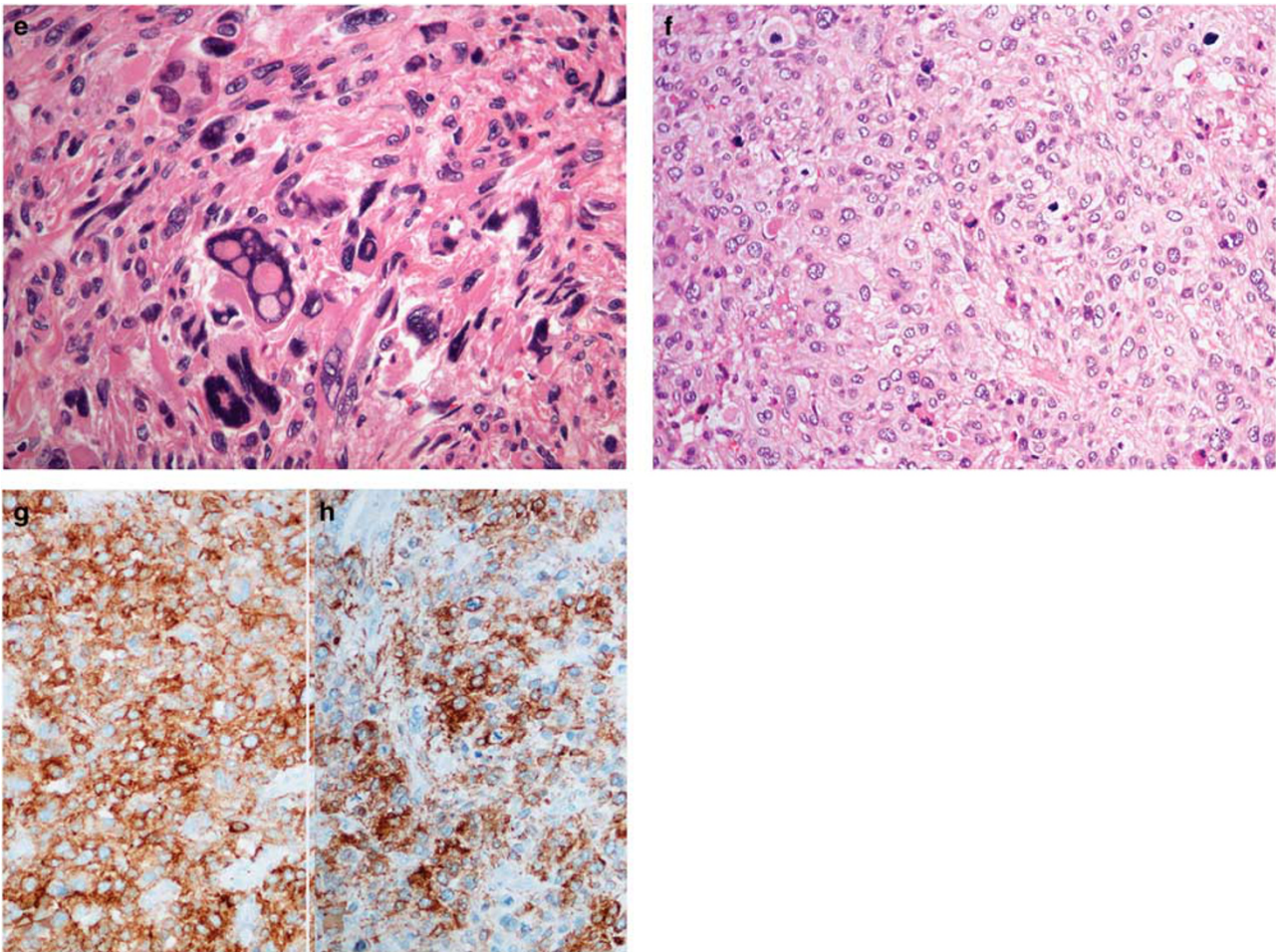


Figure 1 Continued.

focally. The atypical cells were distributed diffusely throughout the lesion. The pleomorphic cells were recognizable at low power examination (10× objectives) (Figure 1d). The nuclei were enlarged with coarse chromatin and occasional pleomorphic nuclei with 'smudged' (degenerative type) chromatin pattern. Nuclear pseudo inclusions were also seen. The cells had abundant eosinophilic cytoplasm (Figure 1f). Mitotic figures ranged from 1 to 5 per 10/hpf (median 2 mitoses/10 hpf). A division figure was considered to be definitive if a mitotic spindle was recognized or hairy or spiky projections emanated from a central chromatin clot. Atypical mitotic figures were not present. Coagulative tumor cell necrosis was absent (Table 2).

Two leiomyosarcomas showed an infiltrative growth pattern (Table 3: pt.4 and 7), two were seen as consult cases, and in these cases the tumor margins could not be evaluated (Table 3: pt.1-2). The remaining leiomyosarcomas did not show infiltrative margins. The vast majority of the analyzed leiomyosarcomas and leiomyosarcoma metastases were composed purely of spindle cells. The spindle cells were arranged in interlacing fascicles, and the nuclei showed moderate to severe nuclear atypia. Nuclear atypia was defined by

the presence of nuclear pleomorphism, nucleomegaly, nuclear membrane irregularity, hyperchromatism, and prominent nucleoli notably at low power, 10× objective (Table 3 and Figure 1f). One leiomyosarcoma showed prominent osteoclastic giant cells (Table 3: pt.2) and another one had epithelioid morphology with clear cell changes (Table 3: pt.3). One leiomyosarcoma (Table 3: pt.4) was associated with an endometrioid adenocarcinoma, diagnosed at the time of hysterectomy. Coagulative tumor cell necrosis defined as necrotic areas with a geographic appearance, atypical ghost cells within the necrosis, sharp interface between viable, and necrotic areas were seen in all cases with one exception (Table 3: pt.5). Mitotic figures ranged from 2 to 43 per 10/hpf (median 25 mitotic figures/10 hpf). All leiomyosarcomas were high-grade (G3) tumors. None of the investigated tumors showed vascular invasion.

Immunohistochemistry

All smooth muscle tumors leiomyomas, leiomyomas with bizarre nuclei and leiomyosarcomas strongly expressed SMA (Figure 1g), and in addition, at least

focal to multifocal Desmin (Figure 1a (inset) and h) and/or h-caldesmon. The leiomyosarcoma with focal epithelioid morphology and clear cell changes (Table 3: pt:3) lacked the nuclear features of melanoma cells, merged with more typical spindled smooth muscle cells and did not express melanocytic markers like S100, HMB45, and Melan A.

Array CGH of Leiomyomas, Leiomyomas with Bizarre Nuclei, and Leiomyosarcomas

In order to assess genomic alterations at a genome-wide level, we performed array-CGH. By comparing the three different entities, a continuous increase of the mean cumulative size of unbalanced genomic regions from leiomyomas (mean 4.7%, range 0–17.1% of aberrant regions, compared to leiomyomas with bizarre nuclei and $P=0.0002$ to leiomyosarcomas) to leiomyomas with bizarre nuclei (mean 14.6%, range 1.2–36.0 of aberrant regions, $P=0.0229$ compared to leiomyosarcomas), and leiomyosarcomas (mean 28.6%, range 1.0–51.9% of aberrant regions) was observed (Figure 2). In order to identify recurrent copy number alterations in the different

tumor types, we subjected our data to the GREVE algorithm. Relative abundance of copy number alterations of leiomyomas, leiomyomas with bizarre nuclei, and leiomyosarcomas are demonstrated in Figure 3. Common changes across all analyzed entities were losses of chromosomes 19q and 22q.

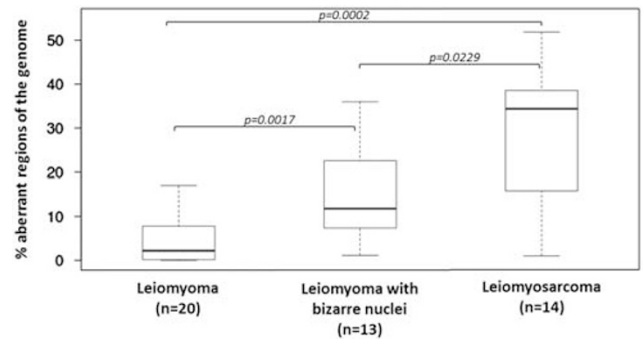


Figure 3 Comparison of the percentage of aberrant genomic regions for leiomyoma, leiomyoma with bizarre nuclei, and leiomyosarcoma. P values were calculated in R using the wilcox.test function.

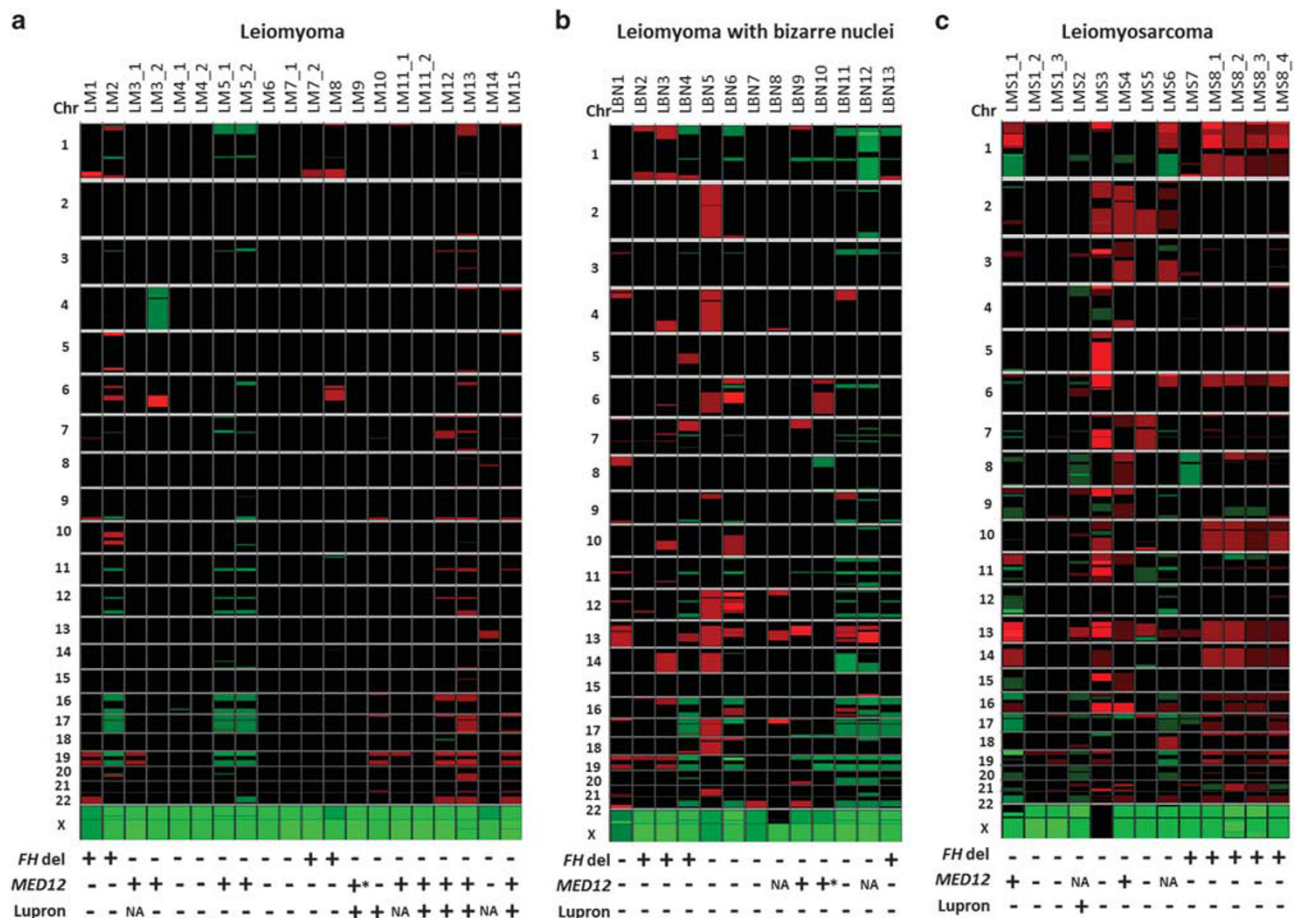


Figure 2 Heat maps of copy number profiles from array-CGH from (a) leiomyoma, (b) leiomyoma with bizarre nuclei, and (c) leiomyosarcoma (black: balanced; red: under-represented; green: over-represented). Presence/absence of *FH* deletions, *MED12* mutations, or a history of Lupron treatment is indicated by +/- . NA, not available.

Changes in Leiomyomas. Copy number alterations were identified in 16 of 20 (80%) leiomyomas, the remaining samples (4/20, 20%) did not harbor any detectable copy number changes. In general a low number of gains and losses were noted. Among leiomyomas the most frequent changes ($\geq 20\%$ of cases) were losses on chromosomes 1q, 7q, 19 and 22, and gains on chromosomes 1q and 16 (Figure 1). The only recurring alteration that was found in $\geq 30\%$ of cases was a loss of chromosome 19 material.

Homozygous losses were found on 1q43 (1/20 cases), high-level amplifications were detected in Xq22.3 (2/20 cases) and in Xp11.3-p11.23 (1/20 cases)

The two cases of leiomyoma with very focal atypia (Table 1: pts. 15, 7b) as well as the cellular leiomyoma (Table 1: pt.14) demonstrated similar genetic changes as those of usual leiomyoma. Indeed the leiomyomas with very focal atypia (Table 1: pt.7b) did not show any genetic changes. Deletions of 1q were not seen (summary see Figure 4a). Six leiomyomas with a history of lupron treatment demonstrated minimal genomic alterations and clustered together with usual leiomyoma without any treatment (Figure 5).

Different tumor samples obtained from one tumor nodule or tumor samples from individual tumor nodules from one patient clustered together, ie, leiomyoma 11_1 and leiomyoma 11_2; leiomyoma 7_1, and leiomyoma 7_2; leiomyoma 4_1 and leiomyoma 4_2 (Table 1) indicating only a limited inter-tumoral and intra-tumoral heterogeneity in a given patient (Figure 5). The exception was pt.3 with 2 samples obtained from different tumors leiomyoma 3_1 and leiomyoma 3_2 (Table 1) that clustered in different branches (Figure 5).

Changes in Leiomyomas with Bizarre Nuclei. All but one leiomyoma with bizarre nuclei (Table 2: pt.7) of the 13 cases of leiomyoma with bizarre nuclei had high number of copy number alterations. The most frequent changes occurring in $\geq 30\%$ of leiomyomas with bizarre nuclei were losses on chromosomes 1q, 13, and gains on chromosomes 1p, 1q, 3p, 7p, 9q, 11q, 16p, 17p, 17q, 19p, 19q, and 22 (Figure 4b).

Homozygous losses were found on 13q14.2-q14.3 (4/13 cases), 13q14.11 (1/13 cases), and 17p13.1, high-level amplifications were found on Xp11.3-p11.23 (5/13 cases), 6p22.1 (1/13 cases), and in 19p.

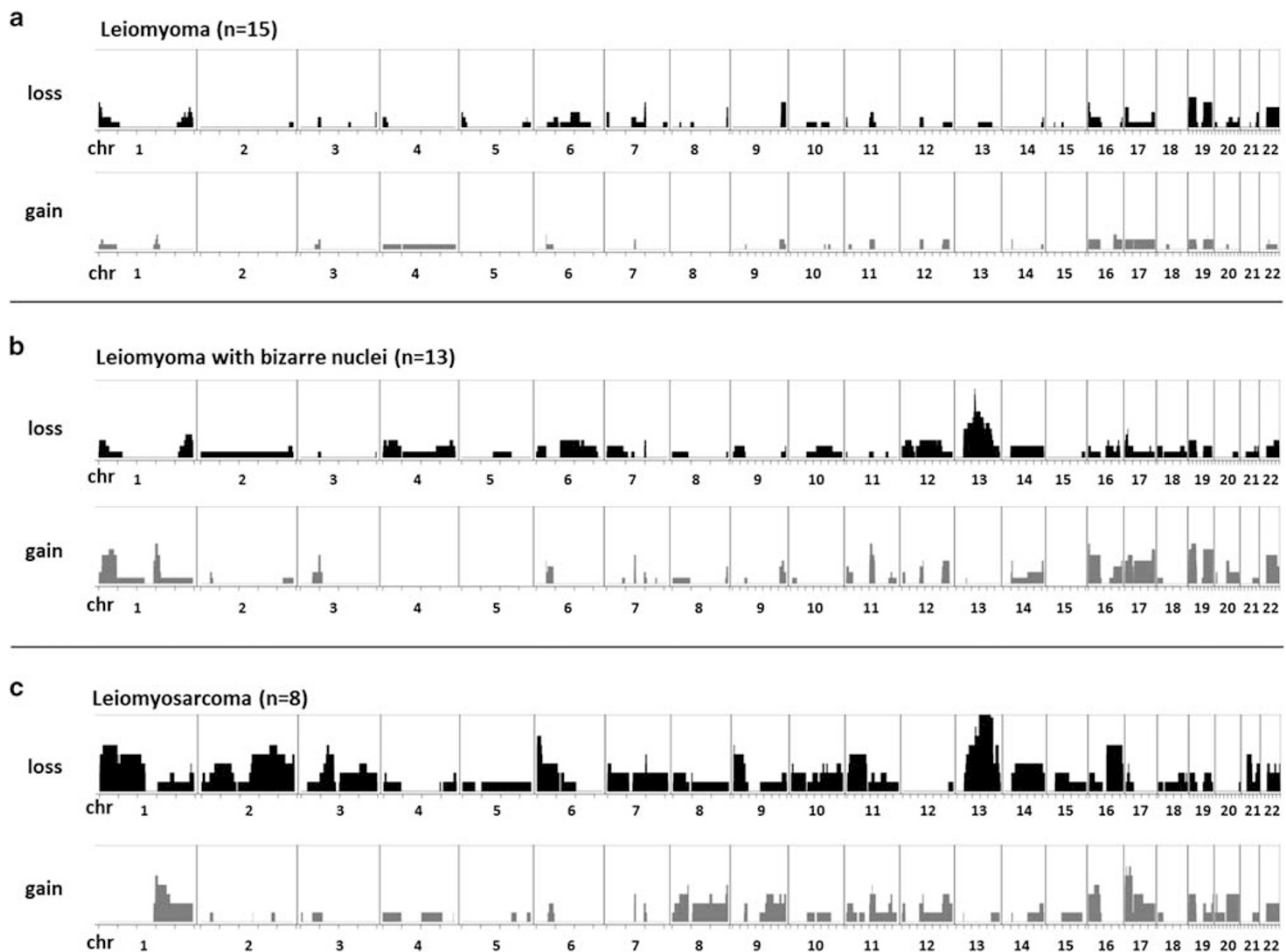


Figure 4 Relative abundance of copy number alteration of (a) leiomyoma, (b) leiomyoma with bizarre nuclei, and (c) leiomyosarcoma calculated with GREVE algorithm. Blue indicates losses of chromosomal regions; red indicates gain of chromosomal regions.

Changes in Leiomyosarcomas. In all 14 leiomyosarcomas multiple gains and losses were identified. Deletions were, in general, more frequent than gains. The most frequent changes occurring in $\geq 30\%$ of cases were losses on chromosomes 1p, 3p, 6p, 7p, 8q, 9p, 10q, 13, 14, 16, 17, 19, 20, and 21, and gains on chromosomes 1q, 7q, 8q, 9q, 11q, 16p, 17q, and 19q (Figure 3). Homozygous losses were detected on 13q14.2-q14.3 (1/14 cases), 1p33 (3/14 cases) 9p24.3 (1/14 cases), 9p21.3 (1/14 cases), 14q24.1 (1/14 cases), Xq11.1-q28 (2/14 cases), 1p43 (2/14 cases), 2q37.3 (1/14 cases), 13q12.11 (1/14 cases), 13q14.2 (1/14 cases), 17p13.3 (1/14 cases), 17p13.2 (1/14 cases), 17p13.1-p12 (1/14 cases), and 22q11.23-q12.1 (1/14 cases), high-level amplifications were found on 4q35.1 (2/14 cases) and on +19p (1/14 cases) (Figure 4c).

By comparing a primary uterine leiomyosarcoma and the subsequent metastases (leiomyosarcoma 1_1, leiomyosarcoma 1_2, leiomyosarcoma 1_3) (Table 3: pt.1), array CGH revealed a high frequency of gains and losses on the primary tumor, whereas the metastases demonstrated limited genetic changes (Figure 4c).

To explore intra-tumoral heterogeneity in a leiomyosarcoma, multiple areas from the tumor (leiomyosarcoma 8_1-leiomyosarcoma 8_4 (Table 3: pt.8) were sampled and analyzed.

Mostly similar copy number alterations were observed in all four samples. However, a distinct pattern at chromosomes 8p, 9q, 11p, 16q, and 21 for leiomyosarcoma 8_2 and leiomyosarcoma 8_4 compared to leiomyosarcoma 8_1 and leiomyosarcoma 8_3 were observed, indicating intra-tumoral heterogeneity (Figure 4c).

By evaluating copy number alterations in smooth muscle tumors, a clear separation of leiomyomas,

leiomyomas with bizarre nuclei, and leiomyosarcomas was not possible. Even in smaller clusters leiomyomas, leiomyomas with bizarre nuclei and leiomyosarcomas branched together suggesting that the three histologically different entities cannot be distinguished based on copy number profiling. However, copy number alterations revealed obvious genetic similarities between leiomyomas with bizarre nuclei and leiomyosarcomas. In general, leiomyosarcomas showed a similar pattern of gains and losses as leiomyomas with bizarre nuclei, with additional copy number alterations and more homozygous losses, and high-level amplifications in leiomyosarcomas (Figure 3).

MED12 Mutations and FH Deletions in Leiomyomas, Leiomyomas with Bizarre Nuclei and Leiomyosarcomas

We sequenced exon 2 of the *MED12* gene, where the most frequently mutated codon 44 is located. In addition, we used array-CGH data to screen for deletions of the *FH* gene located on chromosome 1q43.

Mutations in *MED12* were identified in seven patients with leiomyoma (7/15, 47%), affecting codon 44 in six cases, affecting codon 36 in one, and involved intronic deletion of 10 bp also in one case (Table 1). It is noteworthy that except for leiomyoma 3 (Table 1: pt.3), all tumor samples from the same tumor nodule or from different tumor nodules of an individual patient showed the same mutation. In five patients, two samples from the same tumor (Table 1: pt.11) or two samples from two separate tumor nodules (Table 1: pts. 3, 4, 5, 7) were investigated. In three of those patients, *MED12* mutations were detected. Samples leiomyoma 5 and 11

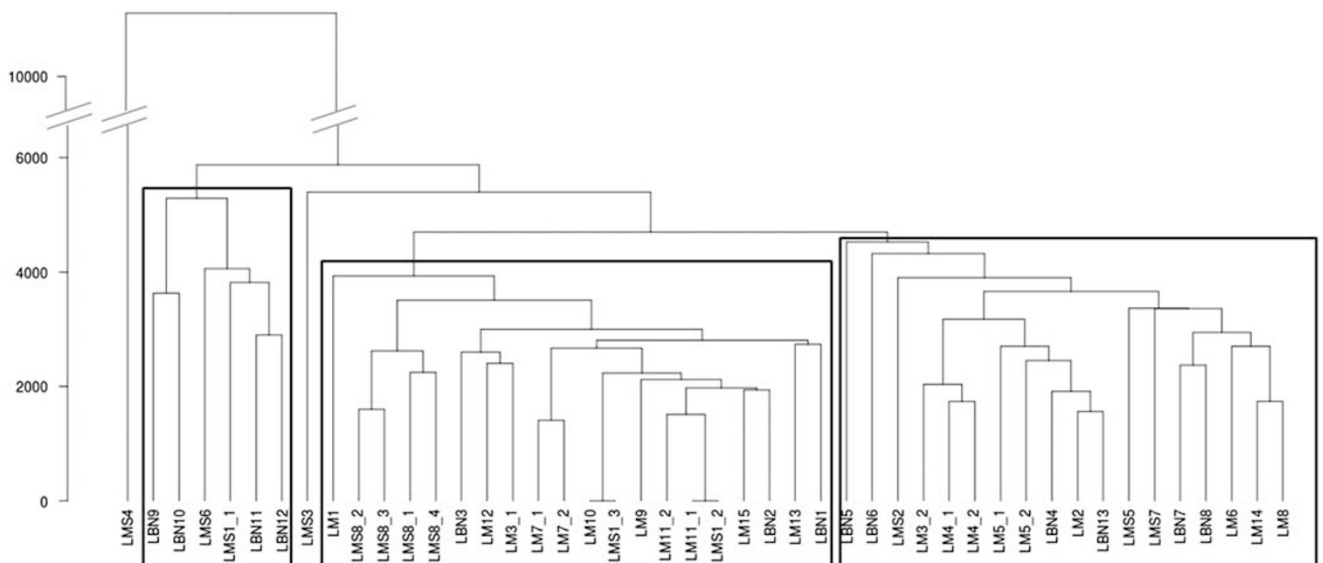


Figure 5 Hierarchical cluster analysis (Manhattan distances of copy number profiles) with leiomyoma, leiomyoma with bizarre nuclei, and leiomyosarcoma.

demonstrated the identical *MED12* mutation (Table 1: pt.5, pt.11), whereas in sample leiomyoma 3 (Table 1: pt.3), two different point mutations affecting the same codon were observed (G44V and G44D) (Table 1: pt.3). Leiomyoma with bizarre nuclei did not show any *MED12* codon 44 hotspot mutations. However, in two patients (15%), a missense mutation of codon 41 (c.122T>C, p.V41A) and a synonymous mutation of codon 52 (c.156T>C, p.(=)), respectively, were observed (Table 2: pts. 9, 10). Only two out of the eight leiomyosarcoma patients (25%) (Table 3: pts.1, 4) showed a codon 44 mutation. Interestingly, in one patient with leiomyosarcoma (Table 3: pt.1) from whom three independent tumor samples (leiomyosarcoma 1_1:primary tumor, leiomyosarcoma 1_2 and leiomyosarcoma 1_3: metastases after 4a and 7a) were available, only the primary tumor showed a *MED12* mutation. In the subsequent metastases, no *MED12* mutation was identified (Table 3: pt.1).

Deletions of the *FH* gene were identified in 4/15 (27%) patients with leiomyomas. In one of these patients (Table 1: pt.7), two independent tumor lesions were available one harboring the deletion, whereas the other lesion did not show any copy number alteration. In leiomyomas with a prior history of lupron therapy, *FH* deletions were not observed.

Furthermore, *FH* deletions were identified in 4/13 patients (30,8%) with leiomyomas with bizarre nuclei (Table 2) and in 2/8 patients (25%) with leiomyosarcomas (Table 3), respectively. All four lesions from the same leiomyosarcoma from one patient showed the deletion (Table 3: pt.8_1-4).

Although a calculation of absolute copy numbers from log₂-ratios is not possible due to varying amounts of tumor cells and the contamination of normal cells, an estimation of *FH* copy numbers revealed that except for one leiomyosarcoma all deletions were heterozygous.

It is noteworthy that *MED12* mutations and *FH* deletion occurred mutually exclusive.

Discussion

Despite recent technical advances, the etiology of uterine smooth muscle tumors has not been established and a molecular fingerprint or biomarkers to reliably distinguish morphologically challenging cases of uterine smooth muscle tumors are not available. Despite tremendous efforts subtyping, uterine smooth muscle tumors continues to rely on purely morphologic criteria including nuclear atypia, number of mitotic figures per 10 hpfs, and tumor cell necrosis.³ The lack of good surrogate markers, presence of unusual histologic features, inter-observer variability, and impact of a variety of hormonal therapies on the morphology of the lesions can complicate clear distinction between these tumors. As a precaution, given the fact that a vast

majority of leiomyosarcomas develop in older patients, a diagnosis of leiomyosarcoma should be made in women up to the end of the fourth decade of life, only after excluding that the increased mitotic activity and/or tumor cell necrosis may have been induced by hormonal therapies. From a cytogenetic point of view, uterine smooth muscle tumors are a very heterogeneous group and driver mechanisms for the development of uterine leiomyoma subtypes, leiomyomas with bizarre nuclei, and leiomyosarcomas remain to be elucidated. Nevertheless, high-throughput sequencing in combination with genetic and cytogenetic data has laid the basis for molecular classification of uterine leiomyomas.^{35,36,42} The only known common oncogenic mechanism shared by benign and malignant smooth muscle tumors are mutations in *MED12* (refs 24–26,31,35) that are frequently detected in uterine leiomyomas and to a much lesser extent in atypical uterine smooth muscle tumors and uterine leiomyosarcomas, respectively.

In addition, it is still under debate whether or not benign smooth muscle tumors of the uterus have the potential for malignant transformation into leiomyosarcoma. In a 2004 study, distinct cytogenetic and molecular genetic pathways were noted for leiomyomas and leiomyosarcomas.⁴³ Therefore, a vast majority of leiomyosarcomas has been presumed to arise *de novo*; progression from leiomyoma to leiomyosarcoma has been considered unlikely. In 2009, a study by Mittal *et al*⁴⁴ raised the possibility that leiomyosarcoma may be derived from leiomyoma.

The primary objective of our study was the assessment of genome-wide copy number alterations and other common molecular changes in a well-characterized group of uterine smooth muscle tumors in order to identify potential surrogate makers that might aid in the diagnosis of these morphologic challenging tumor entities. In addition, a main goal was to explore if there is evidence for transformation from leiomyoma with bizarre nuclei and/or leiomyoma to leiomyosarcoma.

We detected copy number alterations in more than 90% of analyzed samples. However, copy number alteration profiling did not reveal any distinctive pattern that might allow a clear separation between the benign leiomyoma subtypes including classic leiomyoma, hormonally treated leiomyoma, cellular leiomyoma, leiomyoma with bizarre nuclei, and leiomyosarcoma.

In contrast, genetics changes such as losses at chromosomes 19 and 22 were commonly observed across all lesions. This cytogenetic profile was previously found in ~3% of uterine leiomyoma.¹⁹ In a study from Nucci *et al*⁴⁵ pulmonary-based smooth muscle tumors were found to harbor consistent chromosomal aberrations (19q and 22q terminal deletion) in all cases suggesting a key pathogenetic role for sequential tumor suppressor inactivation events. This is in line with our findings

as these specific copy number changes were also found in leiomyomas with bizarre nuclei and leiomyosarcomas indicating that these lesions may share a common pathogenetic mechanism. Furthermore, when we performed the hierarchical cluster analysis, these tumors branched together even in smaller sub-clusters indicating that these histologically different entities cannot be distinguished based on copy number profiling. In concordance with the literature, leiomyosarcomas typically demonstrated the highest frequency of copy number alterations,^{19,29,46} whereas leiomyomas showed a low number of copy number alterations with a few cases (20%) lacking any gains or losses. Interestingly, all cases of leiomyomas with bizarre nuclei and leiomyosarcomas showed a high number of copy number alterations involving nearly all chromosomes. Although leiomyosarcomas showed significantly more copy number alterations with more homozygous losses and higher level of amplifications than leiomyomas with bizarre nuclei, we observed marked similarities in copy number alteration pattern especially between leiomyomas with bizarre nuclei and leiomyosarcomas. A chromosome that was frequently deleted in cases of leiomyomas with bizarre nuclei and leiomyosarcomas was chromosome 13q. In our samples, deletions on chromosome 13q14.2 occurred in 79% of leiomyosarcoma cases and in 54% of leiomyomas with bizarre nuclei. Interestingly, this region harbors the tumor suppressor *RB1*. The involvement of *RB1* in pathogenesis of different malignancies as well as a role for *RB1* in leiomyosarcoma is a frequent reported finding.^{47–49} In contrast, a loss in 13q was only detected in a single leiomyoma indicating a potential use of intact RB as a surrogate marker for leiomyomas. Confirmation of this finding in a larger number and variety of leiomyomas is warranted. Other shared gains and losses between these two subgroups were losses on chromosome 1 and 13, and a gain on chromosome 1q, 7q, 16, 17q, and 20. The most important differences between leiomyomas with bizarre nuclei and leiomyosarcomas were losses on chromosomes 3, 6, 10, 14, and 21, which were frequently found in leiomyosarcomas, but not in leiomyomas with bizarre nuclei.

The overlapping genomic alterations seen in leiomyomas with bizarre nuclei and leiomyosarcomas suggest that these tumors may develop through similar genetic pathways, and that leiomyomas with bizarre nuclei might have a potential to progress into leiomyosarcomas with time and after acquisition of additional alterations.^{3,44,50} It is important to emphasize that despite these common chromosomal alterations leiomyomas with bizarre nuclei are classified as clinically benign smooth muscle tumors rather than 'pre-malignant' conditions. Patients' age at diagnosis greatly differs for leiomyomas with bizarre nuclei and leiomyosarcomas with 41 and 55 years, respectively. Thus, one could suggest a potential time-based relationship, a 'pre-malignant' condition,

would need to progress to cancer.^{31,36,37} However, the vast majority of leiomyoma with bizarre nuclei is treated by hysterectomy and is excised before a putative transformation into clear-cut leiomyosarcoma could occur. Large multicenter studies following patients with leiomyoma with bizarre nuclei over a period of time without surgical treatment to investigate their biology are lacking. The presented findings of copy number alterations in our study challenge the widely accepted concept that leiomyoma with bizarre nuclei, synonym 'pleomorphic/symphastic' leiomyoma (former also known as atypical leiomyoma according to the WHO 2003) is a variant of leiomyoma with marked degenerative cellular (nuclear) changes. On the basis of our findings, the morphologic changes most likely do not reflect just simple 'degenerative' alterations but reflect severe chromosomal abnormalities not seen in usual leiomyomas.

Recently *MED12* mutations have been shown in benign, atypical, and malignant smooth muscle tumors as the first recurrent oncogenic mechanism indicating a general relevance of *MED12* mutations in the pathogenesis of tumors with smooth muscle differentiation.³⁴

Sequencing of exon 2 of the *MED12* gene, where the most frequently mutated codon 44 is located, revealed mutations in 47% of cases in the leiomyoma group in our study. This is in concordance with the literature reporting *MED12* mutations in frequencies between 48 and 92%.³⁶ Our study included an expanded spectrum of leiomyoma subtypes and the results clearly demonstrate that *MED12* mutations can occur in all leiomyoma subtypes investigated irrespective of the tumor size. All diagnostically challenging leiomyomas with a prior history of lupron treatment demonstrated a *MED12* mutation and minimal genomic alterations demonstrating no significant differences to usual leiomyomas.

In contrast to leiomyomas, only 15% of leiomyomas with bizarre nuclei showed mutations in exon 2 of the *MED12* gene. Interestingly, all mutations were located outside the hotspot region suggesting expansion of mutational screening in this subtype might provide further insights.

Furthermore, similar to previous reports where *MED12* mutations were seen infrequently (10–15%) in leiomyosarcomas.^{39,51} Overall, 25% of patients suffering from leiomyosarcomas in our study demonstrated a *MED12* codon 44 mutations in the primary tumor. Surprisingly, in a patient (leiomyosarcoma1) with two subsequent leiomyosarcoma metastases that occurred 4 and 7 years after initial diagnosis *MED12* mutation was no longer detectable in the metastases indicating that a tumor cell clone lacking *MED12* mutation could have metastasized.

Moreover, despite a high frequency of gains and losses in the primary tumor, limited genetic changes were seen in the metastases. Although a lower complexity of genetic changes in metastases

compared to primary tumors has been described in solid tumors,⁵² there are no data available for leiomyosarcomas. One possible explanation could be intra-tumoral heterogeneity suggesting that the metastatic clone was not discovered in the primary specimen as well as the tumor micro-environment.

In our study, *MED12* mutations in leiomyomas with bizarre nuclei and leiomyosarcomas were infrequent and in leiomyomas with bizarre nuclei exclusively found outside of the hotspot areas. Therefore, it seems to be unlikely that *MED12* mutations have a crucial role in possible 'linear progression' of leiomyoma and leiomyoma with bizarre nuclei into leiomyosarcoma. Therefore it is questionable, whether *MED12* acts as a driver mutation in the development of leiomyoma with bizarre nuclei and leiomyosarcoma.

The present study also investigated the presence of *FH* deletion, another proposed driver mutation in leiomyoma. *FH* deletions occurred in 27% of usual leiomyomas, 30.8% of leiomyomas with bizarre nuclei, and 25% of leiomyosarcomas, thus clearly demonstrating another common genetic change in this tumor group. To the best of our knowledge, this is the first report of *FH* deletion occurring in all subtypes of uterine smooth muscle tumors. In addition, the frequency of *FH* deletion in our group of leiomyomas was higher than initially reported.³⁶ In concordance with the literature, *MED12* mutations and *FH* deletions were mutually exclusive in leiomyoma³⁶ as well as leiomyoma with bizarre nuclei and leiomyosarcoma.

In order to investigate inter- and intra-tumoral heterogeneity, we analyzed two or more samples from the same patients and compared the copy number alterations pattern, *MED12* mutations and *FH*-deletions. In leiomyoma group different tumor samples obtained from one tumor nodule or tumor samples from separate tumor nodules from the same patient clustered together indicating only a limited inter-tumoral and intra-tumoral heterogeneity in a given patient. The exception was a single patient with two independent leiomyoma nodules that clustered in different branches and revealed different *MED12* mutations in the same codon (G44V vs G44D). Exploring *FH* deletion in more than one leiomyoma of a patient revealed a case with inter-tumoral heterogeneity. However, exploring inter- and intra-tumoral heterogeneity was limited by the small sample size.

Furthermore, the analysis of individual tumor areas within one large leiomyosarcoma revealed several differences in the copy number alteration profile indicating the presence of an intra-tumoral heterogeneity. Moreover, one patient showed heterogeneity with respect to *FH* deletions. However, due to the limited availability of several samples from the same patients, we were not able to draw any significant conclusions with respect to inter- and intra-tumoral heterogeneity.

In conclusion, our study demonstrates that by evaluating copy number alterations in smooth muscle tumors a clear separation between benign and malignant smooth muscle tumors is not possible. However, copy number alterations revealed marked genetic similarities between leiomyomas with bizarre nuclei and leiomyosarcomas challenging the common concept that a leiomyoma with bizarre nuclei (synonyms: 'pleomorphic/symphastic' leiomyoma and previously termed atypical leiomyoma according to the WHO 2003) is a smooth muscle tumor with degenerative cellular changes and without relationship to leiomyosarcoma. The genetic profile demonstrates that morphologic changes in leiomyomas with bizarre nuclei might not reflect just simple 'degenerative' alterations but rather severe chromosomal abnormalities not seen in usual leiomyomas. Our findings raise the intriguing possibility that leiomyomas with bizarre nuclei may be a precursor of leiomyosarcomas. Our data and findings from two previous studies^{39,51} demonstrate that in contrast to leiomyomas, *MED12* mutations in leiomyomas with bizarre nuclei and leiomyosarcomas do not occur frequently, and that these mutations may not act as 'driver' mutations in the development of leiomyomas with bizarre nuclei and leiomyosarcomas. To further explore the concept of tumor progression and to identify biomarkers to differentiate between diagnostically challenging uterine smooth muscle tumors, high-resolution molecular studies are needed to search for recurrent genetic changes in chromosome areas where overlap between leiomyomas, leiomyomas with bizarre nuclei, and leiomyosarcomas occur.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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