From PTEN loss of expression to RICTOR role in smooth muscle differentiation: complex involvement of the mTOR pathway in leiomyosarcomas and pleomorphic sarcomas

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Over the past decade, comprehensive genomic studies demonstrated that leiomyosarcomas and most of the tumors previously labeled as 'malignant fibrous histiocytomas' share complex karyotypes and genomic profiles, and can be referred to as 'sarcomas with complex genomics'. We recently reported a series of 160 sarcomas with complex genomics such as leiomyosarcomas, myxofibrosarcomas, pleomorphic liposarcomas/ rhabdomyosarcomas and undifferentiated pleomorphic sarcomas. These tumors present with a frequent loss of chromosome 10 region encompassing the tumor suppressor gene _PTEN_. In the present study, we assessed _PTEN_ genomic level and protein expression in this large series of sarcomas with complex genomics, as well as activation of downstream pathways. _PTEN_ partial genomic loss was observed in only 46% of tumors, especially in well-differentiated leiomyosarcomas, whereas up to 68% of these tumors demonstrate a loss of protein expression on western blot analysis. Specific discrepancies in PTEN immunohistochemical results suggested bias in this latter technique. _PTEN_ mutations were rare, with only 4 point mutations in the 65 samples studied. Subsequent activation of AKT and mTOR pathways was only observed in 2 out of 3 of PTEN-deleted tumors. On the other hand, RICTOR, a major component of the mTOR complex 2, was significantly overexpressed in well-differentiated leiomyosarcomas. These results, confirmed on tissue micro-array immunohistochemical analysis of 459 sarcomas, could suggest a link between RICTOR overexpression and leiomyosarcomas oncogenesis. As therapeutics directed against the mTOR pathway are assessed in sarcomas, RICTOR overexpression in sarcomas and its links to therapeutic response need to be assessed.

Over the last decade, major changes have occurred in the classification of soft tissue sarcomas.

Notably, the subgroup of 'malignant fibrous histiocytomas' has been disbanded. Using sharper morphological criteria, most of these poorly differentiated tumors can now be reclassified as poorly differentiated leiomyosarcomas, myxofibrosarcomas, pleomorphic rhabdomyosarcomas and pleomorphic liposarcomas.[1,2] Array comparative genomic hybridization genomic studies sorted out from this group dedifferentiated liposarcomas, characterized by a region of specific amplification encompassing the _MDM2_ and _CDK4_ genes.[3–5] On the whole, only 5% sarcomas can still not be

classified and are now labeled as 'undifferentiated pleomorphic sarcomas'.

These genomic studies also demonstrated that, apart from liposarcomas, most of the previously labeled 'malignant fibrous histiocytomas' with spindle or pleomorphic morphology share complex karyotypes and genomic profiles.[6–8] They can be thus defined as 'sarcomas with complex genomics'. Non-supervised clustering analysis of transcriptomic data among these tumors mostly sorted out smooth muscle differentiation genes in well-differentiated leiomyosarcomas without any conspicuous difference between other histological subtypes.[9–13] These results would suggest common complex biological pathways in the oncogenesis of these tumors.

We recently reported a comprehensive genomic study of a large series of 160 sarcomas with complex genomics.[13] This study allowed us to evidence frequent chromosome 10q deletions in sarcomas with complex genomics; we could also demonstrate that the tumor suppressor gene *PTEN* is significantly underexpressed in 10q-deleted tumors. This gene encodes a dual phosphatase protein that can inhibit AKT signaling, implicated in cell proliferation and resistance to apoptosis.[14] AKT activates the mTOR pathway, and more specifically, the mTOR complex 1 controlling cell growth and metabolism, whereas the mTOR complex 2 involving the protein RICTOR has roles in cytoskeletal organization and AKT activation.[14–16] Both pathways have been implicated in tumorigenesis.[17,18] In our study, we will focus more specifically on *PTEN* gene alterations and protein expression in sarcomas with complex genomics, with emphasis on its consequences in activating downstream pathways.

Material and methods

Tumor Samples

This series included the 167 tumor samples (T1 to T167) of our previous series,[13] plus 12 additional tumor samples (tumors A01 to A12), for which we had performed an array comparative genomic hybridization analysis, but did not have transcriptomic data. Frozen samples and paraffin blocks were obtained from Pathology Departments throughout France, Switzerland and Canada. Experiments were performed in agreement with the Bioethics Law number 2004-800 and the Ethics Charter from the National Institute of Cancer. Histological typing, tumor grade and smooth muscle differentiation were assessed as previously described.[13] Two cell lines were also analyzed, derived from sarcomas with complex genomics samples as previously described.[19] LMS148L is derived from T165, a poorly differentiated leiomyosarcoma with myocardin amplification; S19L is derived from an undifferentiated pleomorphic sarcoma (tumor A05).

Array-Comparative Genomic Hybridization and Transcriptomic Analysis

Genomic DNA extraction and array comparative genomic hybridization experiments were performed as previously described.[13] Total RNAs from tumor samples were analyzed on GeneCHIP Human Genome U133 Plus 2.0 Array (Affymetrix) and normalized as previously described.[13] Hierarchical clustering of tumors T1 to T167 was performed using dChip software (centroid linkage method, rank correlation distance metric, IQR = 2).

Real-Time Genomic Quantitative PCR and Sequencing

To determine the copy number status of *PTEN* and *RICTOR*, real-time PCR was performed on genomic DNA. Primers and probes for *PTEN* were designed in intronic sequences to avoid any cross matching with *PTEN* pseudogene on chromosome 9. The nine exons of the *PTEN* gene were amplified using intron-based primer pairs. Detailed experimental conditions are supplied in Supplementary Information 1.

Western Blot and Immunohistochemistry

Protocols and antibodies used are described in Supplementary Information 1. On western blot analysis, PTEN protein expression was assessed in a semi-quantitative way (0 = no expression, 1 = diminished expression, 2 = normal expression compared with β-actin signal). Expression of other proteins was assessed by the presence or absence of band at expected size on western blot analysis, except for RICTOR and RAPTOR, where intensity of staining was scored from 0 to 3. On immunohistochemistry, PTEN expression was assessed semi-quantitatively from score 0 (absence of expression) to 1 (decreased expression in tumoral cells compared with normal endothelial cells) and 2 (same intensity of staining in tumoral and normal cells). Slides without signal even in endothelial cells, or with 'zone effect' (alternation of entirely positive and entirely negative zones) were considered as 'non-assessable'. RICTOR immunohistochemical expression was assessed by presence or absence of expression and localization of signal.

Tissue micro arrays were assembled in the Institut Bergonié from 459 mesenchymal tumors issued from various French Pathological Departments. Spots of paraffin-embedded tissue (1 mm) were added in triplicates for each tumor. The 459 tumors were therefore represented by 10 paraffin blocks, each block comprising the same 5 internal controls to ensure reproducibility of results.

Statistical Analysis

Comparisons of non-parametric data were established using Fisher's exact test. Pearsons's

correlation factor was used for comparison of quantitative data.

Results

Tumor Samples

The 167 tumor samples previously studied[13] were labeled according to their order in the non-supervised clustering analysis, from T1 to T167. As stated previously,[13] tumors T21 and T22, T35 and T36, T69 and T70, T85 and T86, T138 and T139 correspond to duplicates of the same tumor, from a different DNA extraction sample. Tumor 118 is the local recurrence of tumor 117, and 144 and 145 are different local recurrences of the same tumor. In this study, well-differentiated leiomyosarcomas clusterized together in group A, and were characterized by an over-expression of smooth muscle-related genes. The other cluster groups were differentiated by over-expression of extracellular matrix, adhesion or inflammation genes (groups C to E), or low expression of genes involved in pericentromeric chromosomal region, mitotic spindle and mitosis control (group B). These groups thus encompass various histological subtypes.

Detailed clinical data, as well as array comparative genomic hybridization, Affymetrix data and mutation analysis regarding *PTEN*, *RICTOR* and *RAPTOR* of the clusterized tumors, the additional samples and the cell lines are displayed in Table 1. Briefly, the 179 tumor samples analyzed correspond to 172 patients, presenting with 34 well-differentiated leiomyosarcomas, 21 poorly differentiated leiomyosarcomas, 75 undifferentiated pleomorphic sarcomas, 25 myxofibrosarcomas, 9 pleomorphic liposarcomas, and 8 pleomorphic rhabdomyosarcomas. The mean age at diagnosis was 61 ± 16 years old. The sex ratio was 83 females for 87 males, but significantly unbalanced in well-differentiated leiomyosarcomas from cluster A (21 females for 5 males, $P = 0.0027$). Twenty-one tumors (12%) were retroperitoneal, a proportion also significantly higher in well-differentiated leiomyosarcomas from cluster A ($P < 0.0001$). A total of 68% of the tumors ($n = 110$) were of advanced histological grade, in agreement with their poor differentiation.

PTEN Genomic Level

In our series, *PTEN* array comparative genomic hybridization ratio could be assessed in 111 tumors (Figure 1a). Among them, 60 (54%) displayed a normal *PTEN* array comparative genomic hybridization ratio ($\geqslant 0.8$), whereas 43 others (39%) displayed a partial loss (ratio from 0.5 to 0.8) and 8 (7%) displayed a pronounced *PTEN* loss, with a ratio $\leqslant 0.5$, down to 0.28. *PTEN* partial or complete loss is significantly more frequent in cluster A ($P = 0.003$) and in well-differentiated leiomyosarcomas

($P = 0.033$; Figures 1b and c). *PTEN* genomic copies could be assessed by genomic quantitative PCR in 53 tumors; the results were correlated with array comparative genomic hybridization data ($R = 0.780$).

PTEN mRNA Expression

Exhaustive Affymetrix data are available on the Gene Expression Omnibus database (GSE23980).

Due to the presence of *PTEN* pseudogene on chromosome 9, Affymetrix probe sets for *PTEN* often display a lack of specificity. Two probesets (217492_s_at and 217494_s_at) match both for *PTEN* and *PTENP1*. Some of the most specific probesets, 204054_at and 227469_at, displayed low dynamics in our series; furthermore, they display a partial overlap with *PTENP1* cDNA when blasted on the University of California, Santa Cruz Genome Browser (http://genome.ucsc.edu/). We therefore analyzed *PTEN* expression data using 225363_at, a specific probe set matching the non-translated end of *PTEN* exon 9. As expected, tumors with low array comparative genomic hybridization ratio such as T19, T72 and T93 demonstrate low mRNA expression. For tumors with intermediate or normal array comparative genomic hybridization ratio, there was a good correlation between Affymetrix data and array comparative genomic hybridization ratio ($R = 0.498$), with some notable exceptions, such as cell line S19L that displayed a normal *PTEN* array ratio without any mRNA expression (detailed figures in Table 1).

Sequencing Analysis

A total of 65 tumor samples were thoroughly analyzed for *PTEN* mutations. In 10 additional tumors of B cluster group, only a partial sequencing of *PTEN* gene in exons 6 and 7, and for T40 and T41 in exons 5 and 8, could be assessed, due to a lack of available DNA. Mutations were found in only three tumors. T117 and its local recurrence T118 displayed a punctual deletion in exon 7, with loss of an A base in codon 780, potentially leading to a change in the amino acid sequence from lysine to asparagine on amino acid 260. The frameshift induced would generate a 'stop' codon, truncating the protein at amino acid 265. Tumor A05 displayed a punctual mutation at codon 697 (C > T), potentially generating a 'stop' codon truncating the protein at amino acid 233. This mutation was also found in the cell line derived from the tumor (S19L).

PTEN Protein Expression

Western blot and immunohistochemical results of the 67 tumors assessed for PTEN protein expression are displayed in Table 2.

Table 1 Clinical and genomic data of studied samples

Group	Sample	Diagnosis	Gender	Age	Localization	Grade	PTEN				RICTOR			RAPTOR	
							Mut	CGH	gPCR	Affy.	CGH	gPCR	Affy.	CGH	Affy.
A	T1	UPS	M	40	Subcutaneous	2	WT	0.69	0.24	288	1.03	0.21	991	NA	108
A	T2	WD-LMS	F	72	Thigh	1	WT	NA	0.85	1251	1.01	0.69	580	0.90	27
A	T3	WD-LMS	F	61	Retroperitoneal	2		NA		434	1.25		306	0.91	68
A	T4	WD-LMS	M	49	Thigh	2		NA		1067	1.49		1367	0.70	46
A	T5	WD-LMS	M	48	Vena cava	3	WT	NA		1018	1.31		904	NA	29
A	T6	WD-LMS	F	70	Lung	3	WT	0.63	0.49	1014	1.37	1.26	650	1.06	34
A	T7	WD-LMS	F	74	Retroperitoneal	2	WT	0.65	0.61	508	0.80	1.07	561	1.00	42
A	T8	WD-LMS	F	64	Forearm	2	WT	0.82	0.73	276	1.35	2.11	629	1.07	123
A	T9	WD-LMS	F	70	Axilla	2	WT	NA	0.55	196	1.04	1.61	388	1.00	25
A	T10	WD-LMS	F	55	Thigh	2		NA		748	1.25		553	0.88	89
A	T11	WD-LMS	M	47	Retroperitoneal	2		0.69		677	1.07		749	NA	25
A	T12	WD-LMS	F	29	Thigh	2		NA		707	1.01		305	0.97	54
A	T13	WD-LMS	F	53	Retroperitoneal	1		0.79		917	1.28		1302	0.90	25
A	T14	WD-LMS	F	43	Retroperitoneal	2		NA		1032	1.30		478	0.84	51
A	T15	WD-LMS	F	39	Retroperitoneal	3	WT	0.70	0.67	562	2.79	3.15	2832	0.77	39
A	T16	WD-LMS	F	70	Thorax	3	WT	0.66	0.40	275	0.98	0.89	723	0.75	23
A	T17	WD-LMS	F	45	Retroperitoneal	3		NA		257	NA		1226	1.13	32
A	T18	WD-LMS	F	78	Retroperitoneal	2	WT	0.81	0.73	681	0.95	1.03	514	0.81	34
A	T19	WD-LMS	F	60	Retroperitoneal	3	WT	0.32	0.05	13	1.77	4.18	1200	1.39	66
A	T20	WD-LMS	F	52	Retroperitoneal	3	WT	0.92	0.91	624	1.49	1.68	2487	1.37	143
A	T21	WD-LMS	F	69	Retroperitoneal	3	WT	0.65	0.57	609	1.60	1.75	2250	0.93	52
A	T22	WD-LMS	F	69	Retroperitoneal	3		NA		677	1.64		2607	0.91	44
A	T23	WD-LMS	F	45	Retroperitoneal	3	WT	0.63	0.43	509	1.03	0.72	454	1.27	55
A	T24	WD-LMS	F	47	Retroperitoneal	2		NA		264	1.08		505	0.89	89
A	T25	WD-LMS	F	74	Retroperitoneal	3		0.59		393	1.41		1529	1.08	77
A	T26	WD-LMS	F	50	Retroperitoneal	2	WT	0.86	0.65	505	1.23	1.28	901	1.02	43
A	T27	WD-LMS	M	62	Retroperitoneal	3		0.70		642	0.99		494	0.97	53
A	T28	WD-LMS	M	69	Thigh	2	WT	0.75		532	1.02		1548	0.71	25
B	T29	UPS	F	40	Leg	2	P	NA		979	1.00		287	1.15	42
B	T30	UPS	M	50	Thorax	2	P	NA		808	1.36		342	NA	66
B	T31	UPS	F	26	Thorax	3	P	0.95		1228	1.04		198	1.18	44
B	T32	P-RMS	F	32	Thigh	3	P	NA		480	0.89		196	0.65	22
B	T33	UPS	F	69	Thigh	2	P	1.28		1223	1.31		446	0.64	20
B	T34	WD-LMS	M	50	Perineal	2	WT	0.96		795	1.18		209	0.95	36
B	T35	WD-LMS	M	39	Thigh	1	P	NA		918	1.00		245	0.94	26
B	T36	WD-LMS	M	39	Thigh	1	WT	1.00		1218	0.99		374	1.07	37
B	T37	UPS	M	48	Leg	2	WT	0.99	0.83	690	1.01	0.69	251	0.93	42
B	T38	UPS	M	63	Thorax	3	P	NA		1321	0.99		334	0.94	78
B	T39	UPS	M	58	Knee	2	P	NA		1086	1.31		268	0.87	19
B	T40	P-RMS	M	49	Buttock	2	P	NA		1230	1.04		480	1.30	38
B	T41	UPS	M	58	Retroperitoneal	3	P	NA		1927	0.99		215	0.93	25
C	T42	PD-LMS	F	31	Thigh	3		0.66		869	0.87		302	0.90	24
C	T43	UPS	M	50	Arm	2	WT	0.89	0.70	806	1.42	2.27	605	0.99	30
C	T44	MFS	M	25	Thorax	2	WT	0.86	0.74	776	1.00	1.29	369	1.16	25
C	T45	PD-LMS	F	92	Arm	3		0.82		824	1.14		375	0.93	29
C	T46	UPS	F	83	Thigh	3		NA		1037	1.11		342	0.91	20
C	T47	MFS	M	69	Leg	2		1.06		1709	0.97		333	0.86	25
C	T48	WD-LMS	M	88	Thigh	3		0.77		685	1.08		573	1.02	25

Table 1 Continued

Group	Sample	Diagnosis	Gender	Age	Localization	Grade	PTEN				RICTOR			RAPTOR	
							Mut	CGH	gPCR	Affy.	CGH	gPCR	Affy.	CGH	Affy.
C	T49	WD-LMS	F	36	Shoulder	2		NA		793	1.08		174	0.89	25
C	T50	PD-LMS	F	71	Thigh	2		NA		1175	1.01		710	0.98	26
C	T51	UPS	F	62	Forearm	2		0.94		915	1.15		386	0.96	26
C	T52	UPS	F	52	Thigh	3	WT	0.83	0.89	1520	1.10	0.89	285	1.37	25
C	T53	UPS	M	60	Thigh	3	WT	0.85		695	1.23		297	1.48	34
C	T54	PD-LMS	F	83	Arm	3		1.18		1043	1.32		316	0.96	30
C	T55	PD-LMS	M	16	Thigh	2		NA		1805	0.92		321	0.93	25
C	T56	UPS	F	74	Scapula	3	WT	0.84	0.79	1387	1.00	1.12	239	0.95	30
C	T57	PD-LMS	M	68	Abdomen	3		NA	1.05	804	1.47	1.71	345	1.25	25
C	T58	MFS	M	56	Thigh	3		0.83		589	1.10		529	0.73	25
C	T59	UPS	F	64	Thigh	3	WT	0.86	0.53	360	1.32	0.94	469	1.18	25
C	T60	UPS	M	80	Thorax	3	WT	NANANA	0.64	709	1.07	0.89	346	1.05	25
C	T61	UPS	M	83	Shoulder	NA		0.66		423	1.72		525	NA	25
C	T62	UPS	M	61	Thorax	3		NANA		812	1.25		472	1.11	41
C	T63	UPS	F	64	Thigh	3		0.66		161	1.54		323	1.19	27
C	T64	PD-LMS	F	82	Leg	3		NA		568	1.31		830	1.12	46
C	T65	PD-LMS	F	84	Thorax	3		0.52		417	1.15		209	1.07	26
C	T66	UPS	M	38	Thigh	3	WT	0.36	0.18	534	2.48	3.12	919	1.13	27
C	T67	UPS	M	73	Thigh	3		NA		122	1.40		352	NA	22
C	T68	UPS	M	42	Thigh	3		0.82		888	0.86		479	1.64	35
C	T69	UPS	M	58	Thigh	3		NANA		493	0.94		188	1.16	25
C	T70	UPS	M	58	Thigh	3	WT	0.78	0.62	377	0.98	1.23	219	1.20	22
C	T71	UPS	F	67	Arm	3		NA		647	0.99		308	1.11	25
C	T72	PD-LMS	F	69	Retroperitoneal	NA	WT	0.28	0.09	38	0.83	1.17	171	1.31	44
C	T73	UPS	M	75	Thigh	3	WT	0.97		1064	1.07		162	1.19	25
C	T74	PD-LMS	F	69	Uterus	3	WT	0.96	0.76	1398	1.08	0.74	335	1.25	49
C	T75	PD-LMS	M	71	Thigh	3		NA		1119	0.93		372	1.09	30
C	T76	UPS	M	55	Thigh	3		0.75		867	1.31		387	1.10	27
C	T77	UPS	M	55	Buttock	3	WT	0.70	0.54	1124	0.98	0.95	203	1.21	36
C	T78	WD-LMS	M	69	Thigh	3		NA		904	1.02		617	1.00	25
C	T79	UPS	F	64	Thigh	3		0.39		88	1.25		370	1.01	21
C	T80	UPS	M	72	Thigh	3		NA		884	1.69		553	1.07	23
C	T81	PD-LMS	M	30	Buttock	3	WT	0.73	0.60	916	1.09	1.44	256	1.03	28
C	T82	UPS	M	54	Thigh	3		NA		1593	1.21		508	1.52	38
C	T83	PD-LMS	M	87	Forearm	3		NA		648	1.07		426	1.30	47
C	T84	UPS	F	72	Thigh	NA		0.97		28	1.44		370	0.78	26
C	T85	UPS	F	56	Lung	NA		NA		399	1.69		472	1.05	37
C	T86	UPS	F	56	Lung	NA	WT	0.64	0.83	544	1.81	2.14	393	0.98	25
C	T87	UPS	F	64	Buttock	3		0.65		211	1.14		246	0.81	34
C	T88	PD-LMS	M	58	Thigh	3		NA		811	1.35		605	NA	26
D	T89	UPS	F	67	Thigh	2	WT	0.78	0.76	783	1.13	1.08	811	NA	23
D	T90	UPS	M	33	Shoulder	3		0.76		684	1.15		319	0.99	38
D	T91	P-LPS	M	49	Thigh	2		NA		1935	1.42		478	0.96	28
D	T92	UPS	M	57	Thorax	3		0.77		496	1.06		564	1.12	26
D	T93	UPS	F	53	Thigh	3	WT	0.39	0.25	106	1.12	1.39	432	0.92	25
D	T94	UPS	M	82	Forearm	3		1.15		1180	1.10		546	0.84	28
D	T95	UPS	F	67	Leg	3	WT	1.11	0.95	798	1.13	0.69	731	0.99	25
D	T96	UPS	M	79	Thigh	3		0.78		499	1.48		606	1.02	26

Table 1 Continued

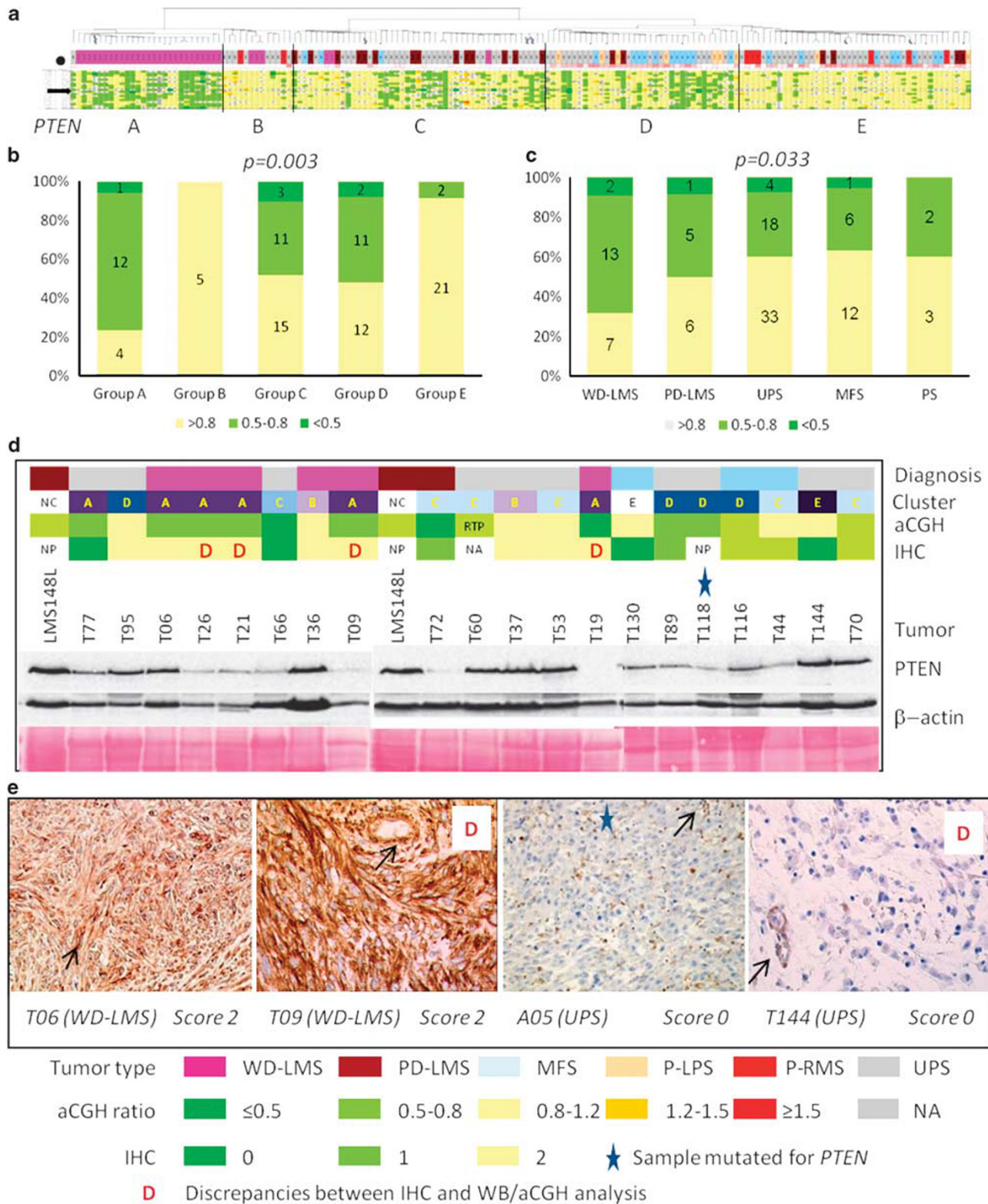| Group	Sample	Diagnosis	Gender	Age	Localization	Grade	PTEN				RICTOR			RAPTOR	
							Mut	CGH	gPCR	Affy.	CGH	gPCR	Affy.	CGH	Affy.
D	T97	UPS	F	68	Thigh	2	WT	0.99	0.90	816	1.67	1.92	724	0.90	19
D	T98	MFS	F	83	Leg	1		1.02		1629	1.04		513	2.29	26
D	T99	P-LPS	F	76	Thigh	3		NA		972	1.30		486	0.92	25
D	T100	UPS	F	41	Leg	3	WT	0.68	0.80	503	1.57	2.00	593	1.09	20
D	T101	PD-LMS	F	38	Thigh	3		NA		1043	1.25		891	1.05	36
D	T102	P-LPS	M	64	Thigh	3		NA		699	1.14		327	0.99	26
D	T103	PD-LMS	F	76	Thigh	3		NA		454	1.02		493	0.44	17
D	T104	UPS	M	78	Thigh	3		NA		1454	1.29		933	0.97	26
D	T105	MFS	M	65	Thigh	2		0.94		535	1.73		1096	1.24	26
D	T106	MFS	M	70	Arm	3		0.77		779	1.68		910	0.99	31
D	T107	MFS	M	56	Thigh	2		1.27		864	1.27		377	NA	25
D	T108	UPS	M	61	Thigh	3		NA		355	1.28		517	1.36	35
D	T109	MFS	F	88	Neck	2		NA		723	1.05		564	0.85	25
D	T110	UPS	F	77	Thigh	3		0.87		488	1.26		436	NA	25
D	T111	P-LPS	M	78	Shoulder	NA		1.04		758	1.56		695	1.37	26
D	T112	MFS	F	77	Thigh	3		0.81		303	1.07		441	0.64	19
D	T113	MFS	F	55	Leg	2		0.81		650	0.99		531	0.98	24
D	T114	MFS	M	63	Hip	NA	WT	0.69	0.59	276	1.01	1.34	452	0.67	26
D	T115	MFS	M	71	Thigh	3	WT	0.81	0.54	458	1.38	1.76	500	0.98	28
D	T116	MFS	M	45	Thigh	2		0.59	0.76	1155	1.58	2.70	645	1.09	27
D	T117	UPS	M	56	Arm	2	MUT	0.32	0.69	180	1.12	2.17	375	1.74	82
D	T118	UPS	M	58	Arm	2	MUT	0.66	0.50	68	0.99	1.29	337	0.93	26
D	T119	UPS	M	74	Thigh	3		0.60		384	1.17		622	1.35	29
D	T120	P-LPS	F	78	Arm	3		0.60		182	1.18		341	1.11	42
D	T121	P-LPS	M	52	Shoulder	3		NA		40	1.03		235	NA	32
D	T122	UPS	M	56	Thigh	2		0.84		590	1.09		580	0.71	16
D	T123	MFS	M	72	Thigh	3		NA		269	1.07		260	NA	26
D	T124	P-LPS	F	62	Thigh	1		NA		976	1.15		329	0.87	23
E	T125	UPS	M	33	Thorax	3		NA		1331	1.06		284	0.91	27
E	T126	P-RMS	M	17	Thigh	3		NA		90	1.55		134	0.99	51
E	T127	P-RMS	M	35	Buttock	3		NA		850	1.24		282	1.03	47
E	T128	P-RMS	M	52	Thigh	3		NA		592	1.73		443	0.87	26
E	T129	UPS	F	57	Thigh	3		NA		803	1.27		487	1.02	32
E	T130	MFS	M	39	Popliteal	3	WT	0.88		576	1.28		942	0.69	19
E	T131	UPS	M	56	Thigh	3		0.81		1368	1.36		421	0.89	26
E	T132	UPS	M	67	Arm	3	WT	NA	0.52	333	1.04	1.36	206	2.06	26
E	T133	UPS	M	66	Thigh	2		0.92		769	1.27		305	1.07	25
E	T134	UPS	F	70	Thigh	3	WT	1.01	0.78	464	1.63	2.41	1135	0.97	19
E	T135	UPS	F	47	Thigh	3		NA		521	1.10		310	1.34	59
E	T136	MFS	F	87	Leg	2		NA		494	1.14		308	1.11	36
E	T137	UPS	M	55	Thigh	3		0.98		480	0.89		154	0.86	30
E	T138	P-LPS	M	46	Arm	3	WT	NA	1.03	688	1.40		187	0.88	25
E	T139	P-LPS	M	46	Arm	3		0.98		568	1.03	1.18	119	1.23	26
E	T140	PD-LMS	F	58	Buttock	3		0.95		550	1.03		303	0.90	18
E	T141	UPS	F	88	Popliteal	3		0.97		604	0.81		303	1.23	32
E	T142	PD-LMS	F	83	Leg	3		NA		718	0.90		265	1.32	26
E	T143	UPS	F	73	Leg	3		0.94		955	1.54		648	1.10	26
E	T144	UPS	F	86	Arm	3	WT	0.99	0.84	737	1.50		573	0.88	20

Table 1 Continued

Group	Sample	Diagnosis	Gender	Age	Localization	Grade	PTEN				RICTOR			RAPTOR	
							Mut	CGH	gPCR	Affy.	CGH	gPCR	Affy.	CGH	Affy.
E	T145	UPS	F	86	Arm	3		1.01		776	1.64	1.50	439	0.95	17
E	T146	UPS	M	82	Arm	3		NA		990	1.05		397	0.97	22
E	T147	UPS	F	78	Thigh	3		NA	0.60	592	1.47	1.69	418	1.08	25
E	T148	UPS	M	74	Forearm	2		1.19		788	1.16		235	UBG	22
E	T149	P-RMS	M	69	Thigh	3		NA		571	1.23		355	1.05	19
E	T150	MFS	F	66	Thorax	2		0.84	0.59	311	1.34	1.19	587	NA	26
E	T151	UPS	M	63	Thorax	3		NA		430	1.03		226	1.09	17
E	T152	UPS	F	65	Leg	3	WT	1.04		750	1.09		279	1.01	25
E	T153	MFS	M	75	Knee	3		NA		780	1.08		382	1.01	26
E	T154	MFS	M	50	Leg	3		1.02		720	1.03		326	0.94	25
E	T155	UPS	M	71	Inguinal	3		NA		264	1.02		248	1.26	26
E	T156	P-RMS	M	69	Scalp	NA	WT	1.10	0.86	457	1.03	0.93	224	0.89	25
E	T157	MFS	M	75	Thigh	2	WT	0.77	0.77	716	1.04	0.82	321	1.01	26
E	T158	UPS	F	79	Forearm	3	WT	0.88	0.73	446	1.27	1.51	502	1.00	26
E	T159	UPS	M	72	Arm	3	WT	0.92	0.99	722	1.02	1.23	508	0.99	26
E	T160	UPS	F	51	Thigh	2		NA		859	1.03		337	0.79	26
E	T161	MFS	F	69	Thigh	3		NA		822	0.98		321	NA	26
E	T162	MFS	M	39	Thigh	3		NA		826	2.08		307	0.94	17
E	T163	P-RMS	M	61	Thigh	2		NA		939	0.96		282	NA	16
E	T164	UPS	F	77	Thigh	3		0.96		700	0.99		150	NA	56
E	T165	PD-LMS	F	82	Forearm	3	WT	0.89	0.80	604	1.00	1.18	345	1.16	25
E	T166	PD-LMS	F	60	Buttock	3	WT	0.82	0.73	1376	1.14	0.93	216	1.12	29
E	T167	P-LPS	M	68	Thigh	3		0.80		777	0.90		197	1.27	34
NC	A01	WD-LMS	F	51	Uterine	3	WT	0.42			0.96			NA	
NC	A02	WD-LMS	F	36	Uterine	2	WT	1.06		910	1.30		904	1.02	26
NC	A03	WD-LMS	F	76	Groin	2	WT	0.70			1.31			1.02	
NC	A04	PD-LMS	M	64	Head and neck	2	WT	0.62	0.44		NA	0.86		NA	
NC	A05	UPS	F	57	Retroperitoneal	3	MUT	1.02	0.91		1.26	0.99		1.27	
NC	A06	UPS	F	61	Retroperitoneal	3	WT	0.75		553	NA		228		26
NC	A07	UPS	M	75	Head and neck	3	WT	0.62			1.09			NA	
NC	A08	UPS	M	74	Leg	NA		1.04			1.30			NA	
NC	A09	UPS			Thorax	NA	WT	0.59			1.39			NA	
NC	A10	MFS			Thigh	3	WT	0.41			1.59			NA	
NC	A11	MFS	M	61	Head and neck	NA	WT	0.76			0.97			1.32	
NC	A12	MFS	M		Arm	3	WT	0.74			0.73			1.19	
CL	LMS148L	PD-LMS	F	82	Forearm	3	WT	0.77		263	1.42		153	1.28	30
CL	S19L	UPS	F	57	Retroperitoneal	3	MUT	1.02		42	NA		34	NA	

Groups: A to E, cluster groups in non-supervised Affymetrix clustering; NC, non-clusterized tumors; CL, non-clusterized cell lines. Diagnosis: WD-LMS, well-differentiated leiomyosarcoma; PD-LMS, poorly-differentiated leiomyosarcoma; UPS, undifferentiated pleiomorphic sarcoma; MFS, myxofibrosarcoma; P-LPS, pleomorphic liposarcoma; P-RMS, pleomorphic rhabdomyosarcoma. Gender: M, male; F, female. Grade: histological grade according to the Fédération Nationale des Centres de Lutte Contre le Cancer. Mut: mutation analysis results; WT, wild type; MUT, mutated sample; P, wild type on the partial sequencing performed; CGH: genomic level assessed by comparative genomic analysis; NA, non-assigned clone; gPCR: genomic real-time quantitative PCR; Affy.: Affymetrix probe sets mean level (*RICTOR* and *RAPTOR*) or specific probe set for *PTEN* (225363_at).

PTEN loss of expression and RICTOR overexpression in soft tissue sarcomas

A total of 57 tumors could be assessed for PTEN protein expression by western blot analysis (representative samples in Figure 1d, as well as their correlation with array comparative genomic hybridization and immunohistochemistry results).

In this series, 38 tumors displayed a partial ($n = 28$, 49%) or complete ($n = 11$; 19%) loss of PTEN expression, whereas the 18 remaining tumors (32%) retained a normal expression of the protein.

Tumor type: WD-LMS, PD-LMS, MFS, P-LPS, P-RMS, UPS

aCGH ratio: ≤ 0.5, 0.5-0.8, 0.8-1.2, 1.2-1.5, ≥ 1.5, NA

IHC: 0, 1, 2, ★ Sample mutated for *PTEN*

D Discrepancies between IHC and WB/aCGH analysis

We could retrieve paraffin blocks from 60 tumors for immunohistochemical analysis. In the 52 tumors where PTEN expression was assessable without zone effects, 56% ($n = 29$) retained normal PTEN expression, whereas the others displayed a partial (17%, $n = 9$) or complete (27%, $n = 14$) loss of expression. Western blot and immunohistochemistry results were concordant in 23 out of 43 cases, with discrepancies in 20 cases, western blot results being more correlated with array comparative genomic hybridization status than immunohistochemistry. Representative samples of concordant and discrepant cases are featured in Figure 1e.

Activated Pathways Downstream of PTEN

To evaluate the impact of PTEN loss of expression in sarcomas with complex genomics, we evaluated the activation of AKT and mTOR pathway by assessment of AKT1- and mTOR-specific phosphorylation on western blot analysis in a large series of tumors (Table 2, representative blots in Supplementary Information 2). AKT1 phosphorylation on serine 473 was evidenced in 17 out of 40 tumors, with a strong signal for five tumors; AKT activation thus occurred in 57% (16 out of 28) tumors displaying partial or complete loss of PTEN expression on western blot analysis. Tumors with AKT activation are preferentially clusterized in cluster A, but the difference was not significant. Subsequent mTOR phosphorylation was observed in 11 AKT-activated tumors. LMS148L and S19L only displayed a faint activation of AKT and mTOR pathways.

Components of the mTOR Complexes: RICTOR Expression

In our series, most tumors displayed a gain of chromosome 5p encompassing *RICTOR*, a gene encoding for the major component of the mTOR complex 2. For each tumor, the mean level of the two array comparative genomic hybridization clones flanking *RICTOR* was correlated with *RICTOR* DNA copy number assessed by genomic quantitative PCR ($R = 0.774$; Table 1). Gains superior to 1.2 in *RICTOR*

region were observed in 75 out of 177 tumors (42%; Figure 2a); however, only a few tumors displayed a strong mRNA expression on Affymetrix data, those tumors mostly corresponding to well-differentiated leiomyosarcomas (Figure 2b) from cluster A (Figure 2c). In comparison, *RAPTOR* gene on chromosome 17 was rarely gained (29 out of 169 tumors, 17%), and its mRNA expression displayed low dynamics.

Of the 35 tumors that were assessed for RICTOR expression on western blot analysis, only 8 (23%) retained a strong signal, all corresponding to well-differentiated leiomyosarcomas from cluster A or B. This was confirmed on immunohistochemistry, as the group A well-differentiated leiomyosarcomas all displayed a strong membranous expression for RICTOR (except T20 that displayed a 'dot-like' paranuclear staining; Figure 2c). Only five tumors that were not well-differentiated leiomyosarcomas displayed RICTOR expression; it was faint and cytoplasmic.

These results were confirmed on a larger series of 459 sarcomas with complex genomics analyzed on tissue micro arrays (exhaustive data in Supplementary Information 3), encompassing 191 leiomyosarcomas, 138 undifferentiated pleomorphic sarcomas, 61 myxofibrosarcomas, 26 pleomorphic sarcomas, 28 dedifferentiated liposarcomas and 15 other sarcomas such as fibrosarcomas or synovial sarcomas. RICTOR was expressed in 91 tumors, its expression was significantly associated with leiomyosarcomas histological subtype ($P < 0.0001$). A total of 77 leiomyosarcomas expressed RICTOR, either in a membranous pattern ($n = 36$), or in a cytoplasmic 'dot-like' pattern ($n = 41$). Absence of RICTOR expression in leiomyosarcomas was preferentially observed in grade 3 tumors that are mostly poorly differentiated ($P < 0.0001$). In the other histological subtypes, the rare positive tumors displayed a diffuse cytoplasmic staining, mostly observed in pleomorphic cells. Of note, all normal vascular smooth muscle cells present in samples displayed a strong membranous and cytoplasmic expression for RICTOR and were used as internal positive controls.

Figure 1 PTEN expression in sarcomas with complex genomics. (**a**) 10q deletion surrounding *PTEN* gene in clusterized sarcomas with complex genomics. In this figure, tumors are named and displayed according to their order in non-supervised clustering of Affymetrix data. On the top of the figure, histological type of each tumor is indicated by a color code in filled squares, described in the bottom of the figure. On the graph, array comparative genomic hybridization ratios are symbolized by color dots according to the cyanine 5/cyanine 3 ratios as stated on the bottom of the figure. This figure is a partial reproduction of the Figure 2 of our previous study: new insights in sarcoma oncogenesis: a comprehensive analysis of a large series of 160 soft tissue sarcomas with complex genomics, Gibault L, *The Journal of Pathology*, 223(1), Copyright 2010 Pathological Society of Great Britain and Ireland; First published by John Wiley & Sons. (**b**) *PTEN* array comparative genomic hybridization ratio according to cluster group. (**c**) *PTEN* array comparative genomic hybridization ratio according to histological type. (**d**) PTEN expression on western blot analysis. Each tumor histological type, *PTEN* array comparative genomic hybridization ratio and PTEN expression on immunohistochemistry is indicated by a color code in filled squares, as defined in the bottom of the figure. (**e**) Representative cases of immunohistochemistry staining for PTEN, compared with normal endothelial cells (arrows): T06 and T09, normal staining (score 2); A05 and T144 (score 0). T09 and T144 demonstrate discrepancies with western blot and genomic comparative hybridization ratio. Abbreviations: NA, non-assessable, NP, non-performed, RTP, genomic level assessed by real-time genomic PCR, D, discrepancies between immunohistochemistry and western blot/array comparative genomic hybridization analysis; WD-LMS, well-differentiated leiomyosarcomas; PD-LMS, poorly differentiated leiomyosarcomas; UPS, undifferentiated pleomorphic sarcomas; MFS, myxofibrosarcomas; P-LPS, pleomorphic liposarcoma; P-RMS, pleomorphic rhabdomyosarcoma.

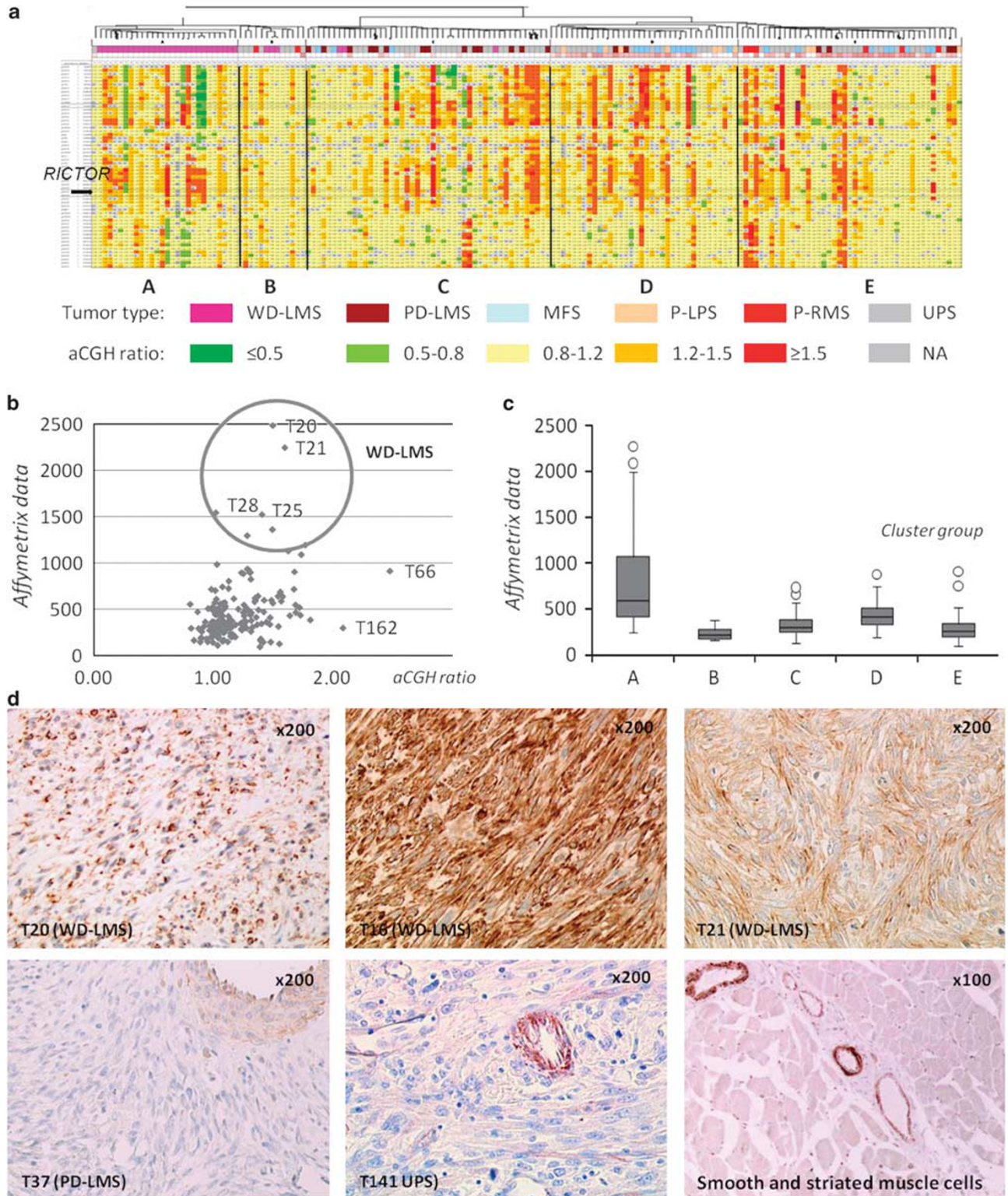**Table 2** Western blot and immunohistochemical analysis

Group	Sample	Diagnosis	Gender	Age	Localization	Grade	PTEN expression						Downstream		RICTOR expression			
							Genomic			Protein			Pathways		Genomic		Protein	
							Mut.	CGH	Affy.	WB	IHC	Discr.	AKT	mTOR	CGH	Affy.	WB	IHC
A	T1	UPS	M	40	Subcutaneous	2	WT	0.69	288	1	na		NA	NA	1.03	991	0	
A	T16	WD-LMS	F	70	Thorax	3	WT	0.66	275		2				0.98	723		MB
A	T19	WD-LMS	F	60	Retroperitoneal	3	WT	0.32	13	0	2	MD	A		1.77	1200		MB
A	T2	WD-LMS	F	72	Thigh	1	WT	NA	1251	2	2	C	NA	NA	1.01	580	2	MB
A	T20	WD-LMS	F	52	Retroperitoneal	3	WT	0.92	624	2	2	C	NA	A	1.49	2487	3	CP
A	T21	WD-LMS	F	69	Retroperitoneal	3	WT	0.65	609	1	2	mD	A	A	1.60	2250	2	MB
A	T23	WD-LMS	F	45	Retroperitoneal	3	WT	0.63	509	1	2	mD	A	A	1.03	454	2	MB
A	T26	WD-LMS	F	50	Retroperitoneal	2	WT	0.86	505	1	2	mD	NA	A	1.23	901	2	MB
A	T28	WD-LMS	M	69	Thigh	2	WT	0.75	532	1	na				1.02	1548		
A	T6	WD-LMS	F	70	Lung	3	WT	0.63	1014	2	2	C	NA		1.37	650		
A	T7	WD-LMS	F	74	Retroperitoneal	2	WT	0.65	508	1	1	C			0.80	561		MB
A	T8	WD-LMS	F	64	Forearm	2	WT	0.82	276	1	2	mD	A	A	1.35	629	2	MB
A	T9	WD-LMS	F	70	Axilla	2	WT	NA	196	0	2	MD	A	A	1.04	388	3	MB
B	T31	UPS	F	26	Thorax	3	P	0.95	1228		2				1.04	198		0
B	T36	WD-LMS	M	39	Thigh	1	WT	1.00	1218	2	2	C	NA	NA	0.99	374	2	MB
B	T37	UPS	M	48	Leg	2	WT	0.99	690	2	2	C	NA	NA	1.01	251	0	0
C	T44	MFS	M	25	Thorax	2	WT	0.86	776	1	1	C			1.00	369		
C	T52	UPS	F	52	Thigh	3	WT	0.83	1520		2				1.10	285		
C	T53	UPS	M	60	Thigh	3	WT	0.85	695	2	2	C		NA	1.23	297	0	0
C	T56	UPS	F	74	Scapula	3	WT	0.84	1387		2				1.00	239		0
C	T57	PD-LMS	M	68	Abdomen	3		NA	804	1	2	mD	NA	NA	1.47	345	0	0
C	T58	MFS	M	56	Thigh	3		0.83	589	2	2	C			1.10	529		0
C	T59	UPS	F	64	Thigh	3	WT	0.86	360	2	2	C			1.32	469		0
C	T60	UPS	M	80	Thorax	3	WT	NA	709	2	2	C	NA	NA	1.07	346	0	CP
C	T66	UPS	M	38	Thigh	3	WT	0.36	534	1	0	mD	A	A	2.48	919	0	0
C	T70	UPS	M	58	Thigh	3	WT	0.78	377	2	na		NA	NA	0.98	219	0	0
C	T72	PD-LMS	F	69	Retroperitoneal	na	WT	0.28	38	0	1	mD	A	NA	0.83	171	0	0
C	T73	UPS	M	75	Thigh	3	WT	0.97	1064		2				1.07	162		
C	T74	PD-LMS	F	69	Uterus	3	WT	0.96	1398	1	na		A	A	1.08	335	0	0
C	T77	UPS	M	55	Buttock	3	WT	0.70	1124	1	0	mD	A	NA	0.98	203	1	0
C	T81	PD-LMS	M	30	Buttock	3	WT	0.73	916	1	1	C	NA	NA	1.09	256		CP
C	T86	UPS	F	56	Lung	na	WT	0.64	544	2	1	mD	A	NA	1.81	393	0	0
C	T87	UPS	F	64	Buttock	3		0.65	211	1	1	C			1.14	246		
D	T100	UPS	F	41	Leg	3	WT	0.68	503	1	2	mD	NA	NA	1.57	593	0	0
D	T114	MFS	M	63	Hip	na	WT	0.69	276	1	1	C	NA	NA	1.01	452	0	CP
D	T115	MFS	M	71	Thigh	3	WT	0.81	458	2	0	MD	A	A	1.38	500	0.5	0
D	T116	MFS	M	45	Thigh	2		0.59	1155	2	2	C			1.58	645		
D	T117	UPS	M	56	Arm	2	MUT	0.32	180	0	0	C	A	A	1.12	375	0	0
D	T118	UPS	M	58	Arm	2	MUT	0.66	68	1					0.99	337		
D	T89	UPS	F	67	Thigh	2	WT	0.78	783	1	1	C	NA	NA	1.13	811	0	0
D	T93	UPS	F	53	Thigh	3	WT	0.39	106	1	0	mD			1.12	432		
D	T95	UPS	F	67	Leg	3	WT	1.11	798	2	2	C	NA	NA	1.13	731	0	0
D	T97	UPS	F	68	Thigh	2	WT	0.99	816	2	na		NA	NA	1.67	724	0	0
E	T130	MFS	M	39	Popliteal	3	WT	0.88	576	1	0	mD	NA	NA	1.28	942		
E	T132	UPS	M	67	Arm	3	WT	NA	333	0			NA	NA	1.04	206		

Table 2 Continued

Group	Sample	Diagnosis	Gender	Age	Localization	Grade	PTEN expression Genomic Mut.	Genomic CGH	Genomic Affy.	Protein WB	Protein IHC	Protein Discr.	Downstream Pathways AKT	Pathways mTOR	RICTOR expression Genomic CGH	Genomic Affy.	Protein WB	Protein IHC
E	T134	UPS	F	70	Thigh	3	WT	1.01	464		2				1.63	1135		MB
E	T138	P-LPS	M	46	Arm	3	WT	na	688	1	1	C			1.40	187		
E	T141	UPS	F	88	Popliteal	3		0.97	604	2	na				0.81	303	1	0
E	T144	UPS	F	86	Arm	3	WT	0.99	737	2	0	MD	NA	NA	1.50	573	0	0
E	T150	MFS	F	66	Thorax	2		0.84	311		2				1.34	587		0
E	T152	UPS	F	65	Leg	3	WT	1.04	750	0	0	C	NA		1.09	279		
E	T158	UPS	F	79	Forearm	3	WT	0.88	446	0			NA	NA	1.27	502	0	0
E	T165	PD-LMS	F	82	Forearm	3	WT	0.89	604	1	0	mD	NA	NA	1.00	345	0	0
E	T166	PD-LMS	F	60	Buttock	3		0.82	1376	2	2	C			1.14	216		0
E	T167	P-LPS	M	68	Thigh	3		0.80	777	1					0.90	197		
NC	A01	WD-LMS	F	51	Uterine	3	WT	0.42			0				0.96			
NC	A02	WD-LMS	F	36	Uterine	2	WT	1.06	910	1	2	mD			1.30	904		MB
NC	A03	WD-LMS	F	76	Groin	2	WT	0.70		0	na				1.31		0	
NC	A04	PD-LMS	M	64	Head and neck	2	WT	0.62		0	0	C	A	NA	na		0	0
NC	A05	UPS	F	57	Retroperitoneal	3	MUT	1.02		1	0	mD	A	A	1.26		1	0
NC	A06	UPS	F	61	Retroperitoneal	3	WT	0.75	553	1	0	mD	NA	NA	na	228	0	0
NC	A07	UPS	M	75	Head and neck	3	WT	0.62		0	na		NA		1.09			
NC	A08	UPS	M	74	Leg			1.04			2				1.30			CP
NC	A09	UPS			Thorax		WT	0.59		1			A	A	1.39			
NC	A10	MFS			Thigh	3	WT	0.41		0	0	C	A	NA	1.59			0
NC	A11	MFS	M	61	Head and neck		WT	0.76		0					0.97			
NC	A12	MFS	M		Arm	3	WT	0.74							0.73			0

Groups: A to E, cluster groups in non-supervised Affymetrix clustering; NC, non-clusterized tumors; CL, non-clusterized cell lines. Diagnosis: WD-LMS, well-differentiated leiomyosarcoma; PD-LMS, poorly differentiated leiomyosarcoma; UPS, undifferentiated pleiomoretroperitonealhic sarcoma; MFS, myxofibrosarcoma; P-LPS, pleomoretroperitonealhic liposarcoma; P-RMS, pleomoretroperitonealhic rhabdomyosarcoma. Gender: M, male; F, female; Age: age at diagnosis. Localization: localization of tumor. Grade: histological grade according to the Fédération Nationale des Centres de Lutte Contre le Cancer; Mut: mutation analysis results; WT, wild type; MUT, mutated sample; P, wild type on the partial sequencing performed; CGH: genomic level assessed by comparative genomic analysis; na, non-assigned clone; gPCR, genomic real-time quantitative PCR; Affy.: Affymetrix probe sets mean level (*RICTOR*) or specific probe set for *PTEN* (225363_at); WB: expression on western blot analysis; 0, absence of expression; 1, diminished expression compared with β-actin signal; 2, normal expression compared with β-actin signal; For RICTOR: intensity of signal assessed from 0 to 3; IHC: immunohistochemistry; 0, absence of PTEN expression in tumoral cells; 1, diminished PTEN expression compared with endothelial cells; 2, normal PTEN expression compared with endothelial cells; na, non assessable due to 'zone effect' technical bias; MB, membranous staining with RICTOR immunohistochemistry; CP, cytoplasmic staining with RICTOR immunohistochemistry; Discr: discrepancies between WB and IHC results; C, concording results; mD, minor discrepancies; MD, major discrepancies; AKT, mTOR: A, activation of the pathway assessed by WB analysis; NA, non-activated pathway assessed by WB analysis.

Discussion

Partial loss of chromosome 10q is a frequent event in sarcomas with complex genomics.[6,13,20–22] Nevertheless, few *PTEN* mutations have been, to date, described in these tumors. Saito *et al*[23] evidenced 2 mutations in a series of 51 sarcomas with complex genomics, in 2 retroperitoneal leiomyosarcomas. The same team demonstrated an immunohistochemical loss of PTEN in 29% of cases in 48 sarcomas; PTEN protein loss of expression nevertheless did not correlate with the genomic alterations.[24] In our

a — Tumor type: WD-LMS, PD-LMS, MFS, P-LPS, P-RMS, UPS. aCGH ratio: ≤0.5, 0.5–0.8, 0.8–1.2, 1.2–1.5, ≥1.5, NA. Cluster groups A, B, C, D, E. RICTOR marked.

b — Affymetrix data vs aCGH ratio scatter plot. WD-LMS cases circled: T20, T21, T28, T25. Other labeled points: T66, T162.

c — Affymetrix data by Cluster group (A, B, C, D, E).

d — Immunohistochemistry (x200 and x100): T20 (WD-LMS), T18 (WD-LMS), T21 (WD-LMS), T37 (PD-LMS), T141 UPS), Smooth and striated muscle cells.

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series of 65 sequenced cases, we could only evidence two different mutations in four samples (three tumors and one cell line derived from a mutated undifferentiated pleomorphic sarcoma), confirming that *PTEN* mutation is a rare event in sarcomas with complex genomics. No mRNA expression was observed in these cases, suggesting an effect of the mutation in mRNA stability. Other mechanisms such as *PTEN* promoter methylation can be discussed in tumors lacking *PTEN* mRNA expression, despite a normal comparative genomic hybridization ratio; this phenomenon is nevertheless also rare in our series. Several studies have suggested that *pten* biallelic deletion is not mandatory to induce a pathological phenotype, supporting the concept of haploinsufficiency in *pten*-deleted murine models.[25–27] This is presumably a frequent event in sarcomas with complex genomics where partial loss of *PTEN* gene occurs in 39% of tumors, with subsequent partial loss of mRNA and protein expression.

PTEN protein expression has, to date, mainly been assessed by immunohistochemistry, using the 6H2.1 monoclonal antibody, described in carcinomas as the most specific, and correlated to genomic data.[28] Yet in our series, discrepancies with western blot and array comparative genomic hybridization results were observed in 20 out of 43 cases. In very rare cases (T115, T130, T144), numerous vessels and endothelial cells retaining PTEN expression in the tumor could potentially explain 'false positive' western blot and comparative genomic hybridization results, hindering PTEN actual loss. On the other hand, some tumors like T19, that are homozygously deleted for *PTEN* and lack mRNA or protein expression on Affymetrix and western blot data, displayed a strong signal on immunohistochemistry, in several independent experiments. This phenomenon was more specifically observed in well-differentiated leiomyosarcomas that all retain a normal immunohistochemical staining, regardless of *PTEN* genomic status. We also observed this phenomenon in two samples of gastrointestinal stromal tumors not included in this series of sarcomas with complex genomics. This could suggest technical bias such as a non-specific cross reactivity of this antibody in these tumors,

maybe with smooth muscle antigens, inciting to a cautious use of this antibody in studies regarding soft tissue sarcomas. Furthermore, immunohistochemistry for PTEN frequently yields to 'zone effect' artifacts, hindering immunohistochemical interpretation, as we observed in eight cases in our series. This problem is even more crucial on tissue micro arrays analysis where tumor samples are of very small size.

The study of the PTEN/AKT/mTOR pathway activation we undertook well reflects the genomic complexity of these sarcomas. Although only one-third retain a normal PTEN expression, several tumors with very low levels of PTEN expression did not yield to downstream activation of AKT and mTOR pathways, except in well-differentiated leiomyosarcomas, where AKT subsequent activation is nearly a constant event in *PTEN*-deleted tumors. Hernando *et al*[29] had already suggested, in a murine model, the implication of the AKT/mTOR complex 1 pathway in the oncogenesis of leiomyosarcomas. This pathway can be blocked by specific inhibitors such as rapamycin.[30] Interestingly, we found that RICTOR, the major component of the mTOR complex 2, is specifically overexpressed in well-differentiated leiomyosarcomas, yielding to a strong membranous or cytoplasmic dot-like signal on immunohistochemistry. As it was also observed on immunohistochemistry in normal smooth muscle cells, we can hypothesize that it may have a potential role in smooth muscle differentiation. Indeed, RICTOR has a role in actin polymerization and cytoskeleton organization.[31] As actin needs to be polymerized to allow smooth muscle genes transcription,[32,33] RICTOR could have a permissive effect on smooth muscle differentiation of well-differentiated leiomyosarcomas. It may also have a role in leiomyosarcomas oncogenic processes, as it is the kinase responsible for AKT1 phosphorylation on serine 473, thus allowing its full activity.[16] RICTOR genomic gains have indeed been linked to tumor recurrence in hepatocellular carcinoma,[34] and its overexpression in glioma cell lines lead to increased anchorage-independent growth and motility.[35]

Thus this study allowed us to demonstrate, in a large series of sarcomas with complex genomics, the

Figure 2 RICTOR expression in sarcomas with complex genomics. (**a**) 5p region and *RICTOR* gene in clusterized sarcomas with complex genomics. In this figure, tumors are named and displayed according to their order in non-supervised clustering of Affymetrix data. On the top of the figure, histological type of each tumor is indicated by a color code in filled squares, described in the bottom of the figure. On the graph, array comparative genomic hybridization ratios are symbolized by color dots according to the cyanine 5 / cyanine 3 ratios as stated on the bottom of the figure. *WD-LMS = Well Differentiated leiomyosarcomas, PD-LMS = Poorly Differentiated leiomyosarcomas, UPS = Undifferentiated Pleomorphic Sarcomas, MFS = Myxofibrosarcomas, P-LPS = Pleomorphic Liposarcoma, P-RMS = Pleomorphic Rhabdomyosarcoma.* This figure is a partial reproduction of the Figure 2 of our previous study: New insights in sarcoma oncogenesis: a comprehensive analysis of a large series of 160 soft tissue sarcomas with complex genomics, Gibault L, *The Journal of Pathology*, 223(1), Copyright 2010 Pathological Society of Great Britain and Ireland. First published by John Wiley & Sons, Ltd. (**b**) *RICTOR* mRNA expression based on the mean level of the Affymetrix probe sets, and its link to the array comparative genomic hybridization ratio. (**c**) Boxplot analysis of *RICTOR* Affymetrix expression in each cluster group. (**d**) RICTOR expression on immunohistochemistry analysis: representative cases. moderate to strong membranous staining in well differentiated leiomyosarcomas such as T16 or T21; 'dot-like' paranuclear staining in T20. Absence of staining in most well differentiated leiomyosarcomas and undifferentiated pleomorphic sarcomas (T37, T141), whereas normal smooth muscle cells demonstrate a strong staining in all samples.

frequent loss of PTEN expression in these tumors, presumably by a 'gene-dose effect' mechanism. Subsequent AKT and mTOR activation is nevertheless inconstant. Furthermore, immunohistochemistry for PTEN demonstrated technical bias, especially in leiomyosarcomas, precluding its use in pre-therapeutic assessment in these tumors. Indeed, in a small series of temsirolimus-treated leiomyosarcomas, we could not predict therapeutical response by PTEN immunohistochemical assessment.[36] Interestingly, our results also point out that the mTOR complex 2 component RICTOR, yet scarcely studied, could have a major role in leiomyosarcomas oncogenesis. As therapeutics using mTOR inhibitors are being assessed in soft tissue sarcomas,[36–39] further comprehensive studies are needed to determine which subgroups of patients would benefit more from such therapeutics. Additional simple predictive tools are needed to assess activation of mTOR pathway in soft tissue sarcomas, as well as RICTOR overexpression and their potential influence in the therapeutic response.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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