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RESEARCH ARTICLE

Distinct molecular subtypes of uterine leiomyosarcoma respond differently to chemotherapy treatment

Yang An[1,2†], Shuzhen Wang[1,2†], Songlin Li[1,3], Lulu Zhang[1,3], Dayong Wang[4], Haojie Wang[1,2], Shibai Zhu[5], Wan Zhu[6], Yongqiang Li[1,2], Wenwu Chen[3], Shaoping Ji[1,2*] and Xiangqian Guo[1,2,7,8*]

Abstract

Background: Uterine leiomyosarcoma (ULMS) is an aggressive form of soft tissue tumors. The molecular heterogeneity and pathogenesis of ULMS are not well understood.

Methods: Expression profiling data were used to determine the possibility and optimal number of ULMS molecular subtypes. Next, clinicopathological characters and molecular pathways were analyzed in each subtype to prospect the clinical applications and progression mechanisms of ULMS.

Results: Two distinct molecular subtypes of ULMS were defined based on different gene expression signatures. Subtype I ULMS recapitulated low-grade ULMS, the gene expression pattern of which resembled normal smooth muscle cells, characterized by overexpression of smooth muscle function genes such as *LMOD1*, *SLMAP*, *MYLK*, *MYH11*. In contrast, subtype II ULMS recapitulated high-grade ULMS with higher tumor weight and invasion rate, and was characterized by overexpression of genes involved in the pathway of epithelial to mesenchymal transition and tumorigenesis, such as *CDK6*, *MAPK13* and *HOXA1*.

Conclusions: We identified two distinct molecular subtypes of ULMS responding differently to chemotherapy treatment. Our findings provide a better understanding of ULMS intrinsic molecular subtypes, and will potentially facilitate the development of subtype-specific diagnosis biomarkers and therapy strategies for these tumors.

Keywords: Uterine leiomyosarcoma, Molecular subtype, Molecular signature, Gene expression pattern, Subtype-specific treatment

Background

Uterine leiomyosarcoma (ULMS) is a type of malignant soft tissue tumors showing distinctive morphologic features and molecular signatures [1]. ULMS has poor prognosis and high recurrence rate [2–4]. Currently, the treatment of ULMS is mostly by surgery with some adjuvant therapies, such as cytotoxic chemotherapy and radiotherapy [5–7]. Due to the complex molecular heterogeneity of ULMS and unavailability of targeted therapeutic methods, the

five-year survival rate of ULMS is still low [8]. By gene expression profiling methods, a number of malignant tumors, including breast cancer, gastric cancer, and uterine carcinosarcoma [9–13], have been categorized into different molecular subtypes. Based on the subtype information, patients may be able to receive better diagnosis and more effective therapeutic options [14]. Thus, it is important to classify molecular subgroups of ULMS, which may provide a better understanding of disease mechanism and guide future precision treatment. Previously, we analyzed leiomyosarcoma (LMS) cases from uterine and extra-uterine sites to classify molecular subtypes [15]. Nevertheless, the treatments for LMS were different regarding LMS locations, which were between uterine and extra-

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* Correspondence: shaopingji@henu.edu.cn; xqguo@henu.edu.cn
†Equal contributors
[1]Department of Biochemistry and Molecular Biology, Joint National Laboratory for Antibody Drug Engineering, Institute of Biomedical Informatics, Medical School, Henan University, Kaifeng 475004, China
Full list of author information is available at the end of the article

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uterine LMS patients in clinical practice. Italiano et al. demonstrated the molecular heterogeneity of LMS from extra-uterus by genetic profiling [16]. But the molecular heterogeneity of ULMS was less investigated until now.

In this study, by analyzing gene expression data sets, we identified and defined two molecular subtypes of ULMS, each of which presents subtype-specific gene expression patterns. Genes and pathways enriched in subtype I ULMS were associated with smooth muscle function, while genes and pathways involved in epithelial to mesenchymal transition (EMT) and tumorigenesis were enriched in subtype II ULMS. Our findings will provide a better understanding of ULMS pathogenesis and facilitate the development of more effective and individualized therapies.

Methods

Bioinformatic analyses and immunohistochemistry staining

To identify the molecular subtypes of ULMS, we collected and analyzed expression profile data sets from TCGA database and determined the optimal number of molecular subtypes of ULMS by Consensus Clustering (R package, ConsensusClusteringPlus [17]). The accuracy of subtype assignments was evaluated by Silhouette analysis (R package cluster [18]). The subtype-specific gene expression patterns were investigated by Gene Set Enrichment Analysis (GSEA) and Significance Analysis of Microarrays (SAM-seq). To identify the pathways that enriched in each subtype,

the KEGG pathway analysis was performed online (https://david.ncifcrf.gov/). Cluster 3.0 and TreeView were used to perform hierarchical clustering to view the top 500 significantly over-expressed genes from each subtype. To develop subtype specific diagnostic biomarkers, genes overexpressed in subtype I (*LMOD1*, 1:20, Sigma, CAT#HPA028325) and subtype II (*ARL4C*, 1:120, Sigma, CAT#HPA028927) ULMS were selected for immunohistochemistry staining (IHC) based on SAM-seq result and the antibody availability. The procedure and scoring of IHC were performed as described previously [15].

Statistical analyses

Statistical significance was assessed by the chi-square and Fisher exact tests. For all statistical analyses, p value less than 0.05 was considered statistically significant.

Results

Consensus clustering of gene expression profiles revealed two molecular subtypes of uterine leiomyosarcoma

Level 3 RNAseq expression data of 29 ULMS cases were collected from The Cancer Genome Atlas (TCGA) and used to determine the molecular heterogeneity of ULMS by consensus clustering (Fig. 1a), a method that estimates cluster stability by iterative resampling of genes and samples [17]. The consensus clustering demonstrated that two subtypes were the optimal number for ULMS, as indicated by the empirical cumulative distribution plots, showing the
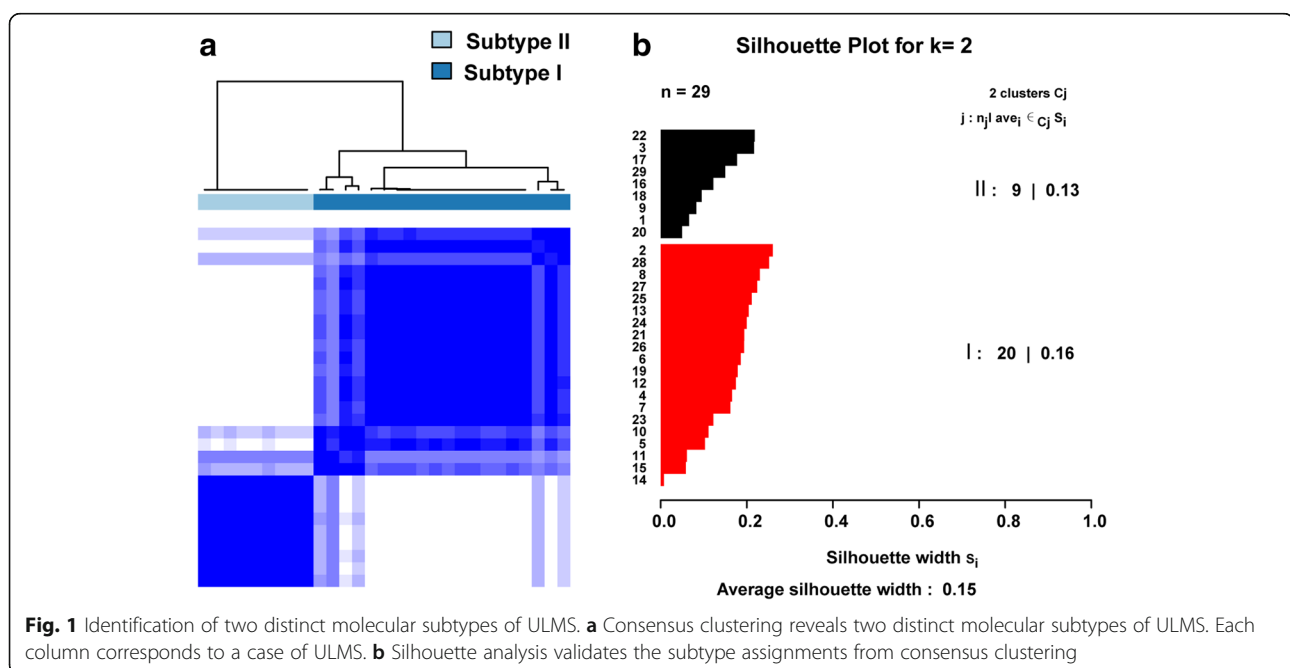

Fig. 1 Identification of two distinct molecular subtypes of ULMS. **a** Consensus clustering reveals two distinct molecular subtypes of ULMS. Each column corresponds to a case of ULMS. **b** Silhouette analysis validates the subtype assignments from consensus clustering

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greatest increase in the area under CDF curve (Additional file 1: Figure S1A and B). Next, the confidence of subtype assignment from Consensus Clustering was evaluated by silhouette analysis (Fig. 1b), which showed that all cases from both subtypes have a positive silhouette value, confirming the two molecular ULMS subtypes.

Clinicopathologic features of ULMS molecular subtypes

Next, we compared the clinicopathologic features between subtype I and subtype II ULMS patients. As shown in Table 1, the ULMS subtype is significantly associated with clinical treatment response. Specifically, subtype I patients were significantly more responded to chemotherapy treatment than subtype II. However, there is no significant association between molecular subtypes with other clinicopathologic characteristics, including tumor weight, metastasis status, invasion and necrosis (Table 1).

Distinct molecular subtypes of ULMS have different gene expression patterns

We next investigated the subtype-specific gene expression patterns of ULMS by Gene Set Enrichment Analysis (GSEA) [19]. GSEA analysis showed that 2669 gene sets were enriched. Among the 2669 gene sets, 1568 gene sets were over-expressed in subtype II while the other 1101 gene sets were over-expressed in subtype I (Fig. 2a). Gene sets associated with leiomyosarcoma and myogenic targets were enriched in subtype I (Fig. 2b), whereas gene sets involved in EMT and tumorigenesis were enriched in subtype II (Fig. 2c). Interestingly, genes over-expressed in subtype II were also associated with cell cycle, proliferation, organ development and

Table 1 Clinicopathologic characteristics ($N = 29$)

	patients, n (%)	Subtype I	Subtype II	*p* value
Age (year)				
Mean	56	54	60	
Range	35–73	35–73	41–71	
Race				
White	20	14	6	
Black or African American	4	2	2	
Asian	1	1	0	
Unknown	4	3	1	
Tumor weight				0.4
Mean	341.83	303.8	426.33	
Range	55–1100	58–1100	55–1077	
Treatment best response				0.04
Complete Response	8(27%)	6	2	
Partial Response	2(7%)	0	2	
Clinical Progressive Disease	4(14%)	4	0	
Unknown	15(52%)	10	5	
Metastatic disease confirmed				0.18
YES	10 (34%)	5	5	
NO	11 (38%)	9	2	
Unknown	8 (28%)	6	2	
Contiguous organ invaded				0.24
YES	4(14%)	2	2	
NO	8 (28%)	7	1	
Unknown	17 (58%)	11	6	
Necrosis percent				0.52
Mean	20.33	23.5	14	
Range	0–50	1–50	0–33	

* $p < 0.05$

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tumorigenesis. These genes include *CDK6, BMP1, MAPK13, PDGFRL* and *HOXA1* (Fig. 3). Subtype I ULMS was enriched with genes involved in smooth muscle function (Fig. 3), including *LMOD1, SLMAP, MYLK,* and *MYH11,* all of which are the smooth muscle-specific markers [20–22].

To further explore the different gene expression patterns of the two subtypes of ULMS, we performed Significance Analysis by SAM-seq. Our results showed that 1947 genes were significantly differently expressed between two subtypes. Among these genes, 1050 were over-expressed in subtype II ULMS while 897 genes were over-expressed in subtype I. Next, we analyzed the top 500 over-expressed genes in each subtype by hierarchical clustering. As the heatmap shows, those genes were significantly over-expressed in subtype I and subtype II, respectively (Additional file 2: Figure S2). Consistent with the GSEA results, the pathways enriched in subtype I were associated with smooth muscle function, such as vascular smooth muscle contraction, calcium signaling pathway, and regulation of actin cytoskeleton (Table 2). Pathways involved in tumorigenesis associated with subtype II,
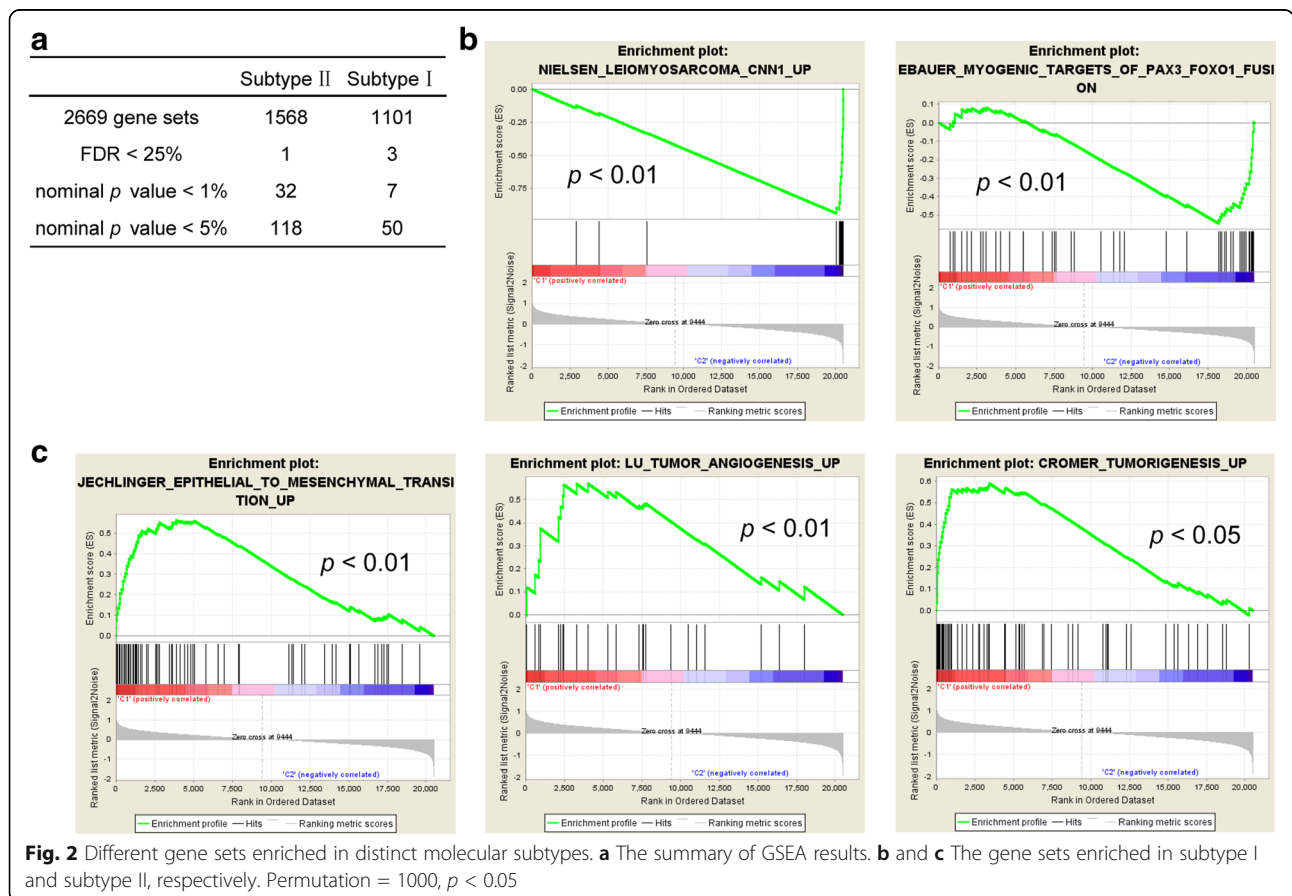
such as pathways in cancer, TGF-β and Hedgehog signaling pathway (Table 2).

Immunohistochemistry staining of ULMS subtype specific biomarkers

To further validate ULMS subtypes and develop subtype specific diagnostic biomarkers, we selected ULMS subtype specific genes for IHC staining by SAM-seq result and availability of commercial antibodies. The IHC results showed that subtype I biomarker LMOD1 was positively stained in 51 of 68 ULMS cases (75%), while 13 subtype II ULMS cases were positive for ARL4C (13/68, 19%) (Fig. 4a), and the correlation coefficient of staining results of these two genes across all the ULMS cases is –0.43 (Fig. 4b).

Discussion

Uterine sarcomas are composed of leiomyosarcoma, endometrial stromal sarcoma and carcinosarcoma. Among these, leiomyosarcoma is the most common subclass, mainly found in postmenopausal women [1, 23]. Although early diagnosis could improve the survival rate of ULMS patients, there are still challenges for treating late stage ULMS patients due to its high
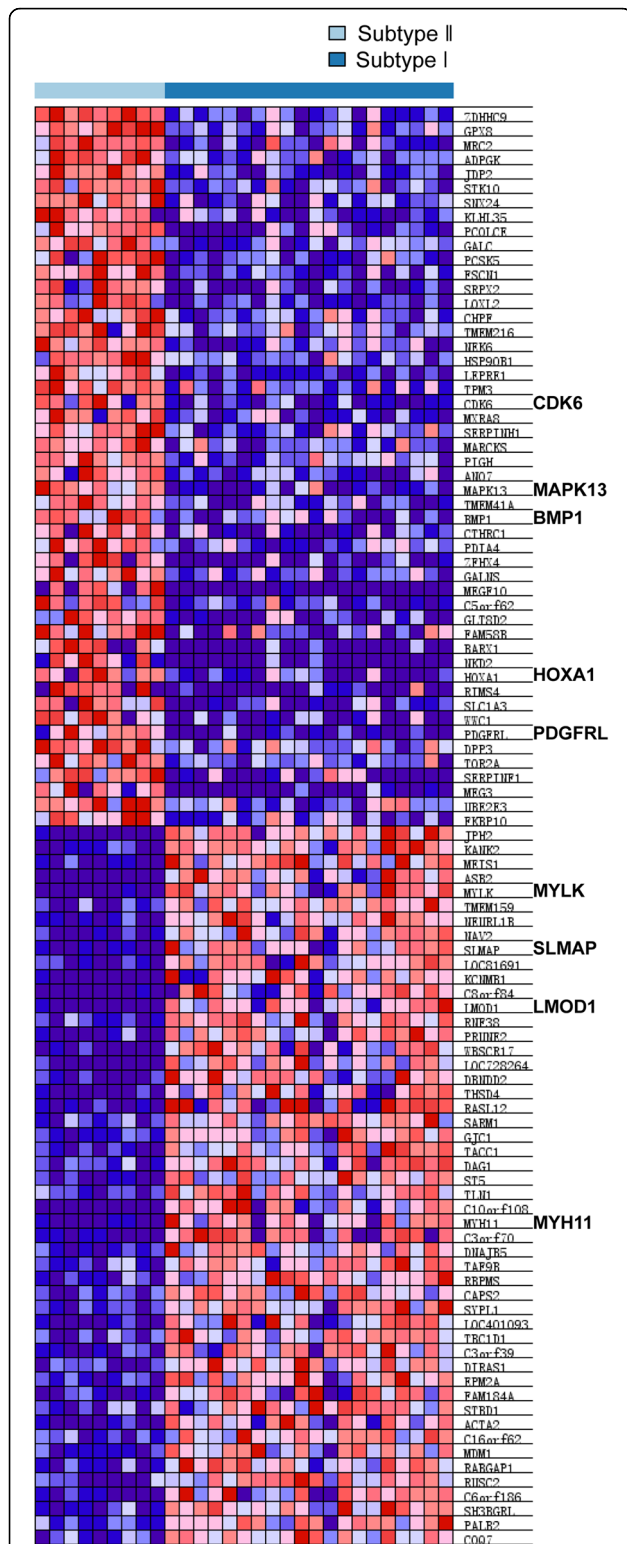

Fig. 2 Different gene sets enriched in distinct molecular subtypes. **a** The summary of GSEA results. **b** and **c** The gene sets enriched in subtype I and subtype II, respectively. Permutation = 1000, *p* < 0.05

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Fig. 3 Different gene expression signatures enriched in distinct molecular subtypes. Subtype I and subtype II ULMSs have different gene expression signatures revealed by GSEA. Each row denotes a gene and each column corresponds to a case of ULMS. Red, over-expressed genes; Blue, down-expressed genes
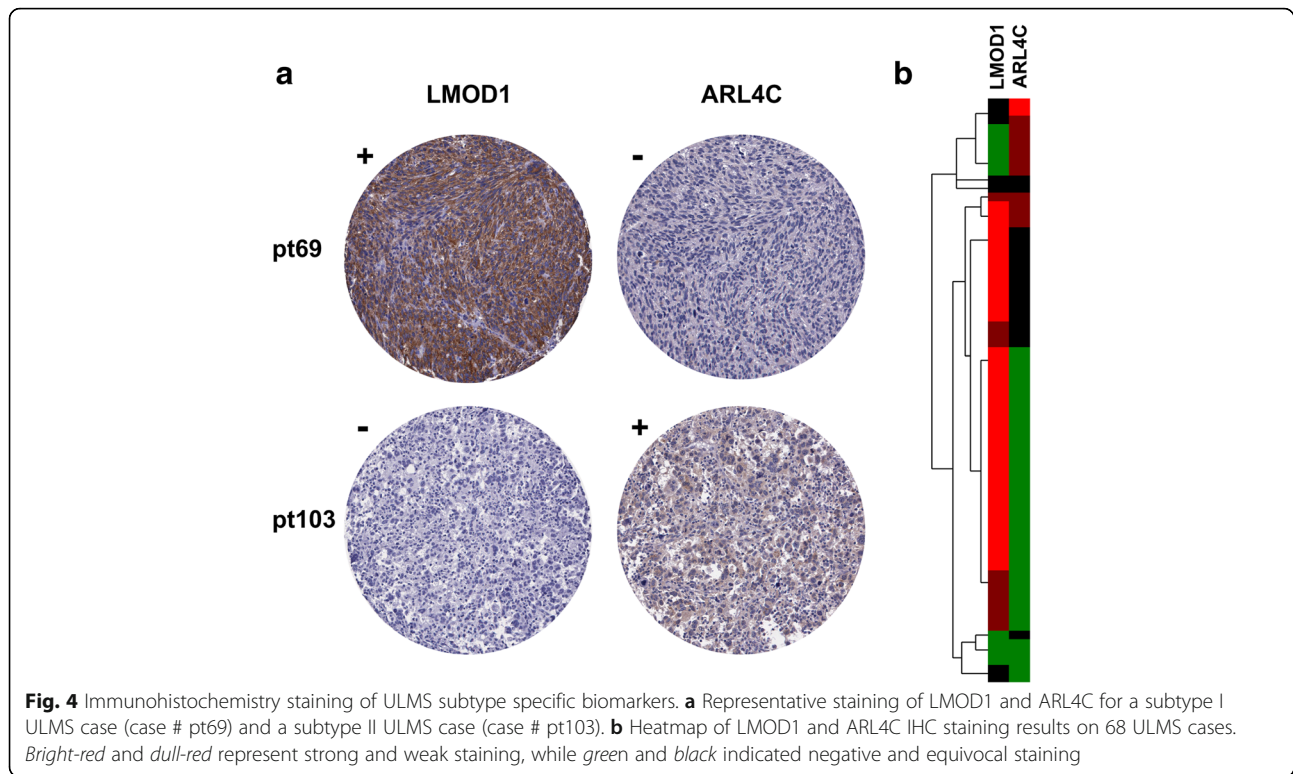
Table 2 Pathways enriched in each molecular subtype

	Pathways	*p* Value
Subtype I	Vascular smooth muscle contraction	7.2E-08
	Focal adhesion	0.00021
	Calcium signaling pathway	0.00497
	Regulation of actin cytoskeleton	0.00702
Subtype II	Ribosome	1.7E-05
	Hedgehog signaling pathway	1.28E-02
	Pathways in cancer	0.02433
	TGF-beta signaling pathway	0.03385

invasiveness and relatively high resistance to radio-therapy and chemotherapy [24]. Molecular subtyping of tumors based on their gene expression profiling have guided subtype-specific diagnosis, prognosis, and aided to develop subtype targeted therapies [17]. In our study, we identified two molecular subtypes of ULMS and found that these two subtypes exhibited significantly different gene expression patterns and distinct sensitivities to chemotherapy treatment. Nevertheless, please kindly noted that the number of patients with clinical treatment response information in this study is low, and additional large scale valid-ation of treatment responses on distinct molecular subtypes will be needed.

Among the genes and pathways enriched, subtype I ULMS showed overexpression of smooth muscle-specific markers, including *LMOD1*, *SLMAP*, *MYLK*, *MYH11*. LMOD1, also known as Leiomodin-1, could be activated by serum response factor (SRF) or myocardin (MYOCD) and functions in smooth muscle cell differentiation [20]. SLMAP, or sarcolemmal membrane-associated protein, is involved in micro-tubule organization [25], excitation-contraction coupling [26] and myoblast fusion [27]. Myosin light chain kinase and Myosin-11 protein, encoded by *MYLK* and *MYH11*, respectively, are components of smooth muscle cells SMC contractile apparatus [28]. MYLK is a Ca^{2+}/CaM-dependent kinase and involved in smooth muscle contraction by promoting the inter-action between myosin and actin filaments [29]. Myosin-11 belongs to the myosin heavy chain family, and has contractile activity by hydrolyzing ATP [30].

Subtype II ULMS showed overexpression of genes enriched in pathways including cancer, TGF-β and Hedgehog signaling. Particularly, over-expression was found in the following genes, including *CDK6*, *MAPK13* and *HOXA1*. CDK6 (Cyclin-dependent kin-ase 6) is a cell cycle regulator and forms a complex with cyclin D to initiate G1 to S phase transition by phosphorylating and inactivating Rb [31–33]. *MAPK13* (Mitogen-activated protein kinase 13)

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Fig. 4 Immunohistochemistry staining of ULMS subtype specific biomarkers. **a** Representative staining of LMOD1 and ARL4C for a subtype I ULMS case (case # pt69) and a subtype II ULMS case (case # pt103). **b** Heatmap of LMOD1 and ARL4C IHC staining results on 68 ULMS cases. *Bright-red* and *dull-red* represent strong and weak staining, while *green* and *black* indicated negative and equivocal staining

belongs to the MAP kinase family and functions in cell proliferation, differentiation and development. *MAPK13* is also involved in cell motility and invasion, serving as a diagnostic marker for cholangiocarcinoma [34]. *MAPK13* is highly expressed in uterine and ovary tumor tissues, especially in gynecological cancer stem cells, and has tumor-initiating activity that was involved in tumorigenesis [35]. Highly expressed in many types of tumor cells, *HOXA1* (Homeobox A1) is a DNA-binding protein and involved in facilitating cell proliferation, invasion, metastasis, and tumor progression [36, 37]. These proto-oncogenes were all over-expressed in subtype II, suggesting that the extent of malignancy of subtype II ULMS may be higher than that of subtype I, and subtype II may represent high-grade ULMS.

Conclusions

In conclusion, we characterized distinct intrinsic molecular subtypes of ULMS with different gene signatures. Our findings provide new insights into the understanding of the pathogenesis of ULMS, facilitate the development of subtype-specific diagnostic biomarkers and targeted treatment for ULMS. Furthermore, our finding may provide valuable information to develop individualized medicine for ULMS patients.

Additional files

Additional file 1: Figure S1. Delineation of two distinct molecular subtypes of ULMS. (A) Empirical cumulative distribution plots. (B) The increased area under the CDF curve along with increased number of molecular subtypes. (TIFF 215 kb)

Additional file 2: Figure S2. Heatmap of the top 500 genes over-expressed in distinct molecular subtypes. Based on SAM-seq result, the top 500 genes over-expressed in each subtype were selected to form the TOP500 genes. Hierarchical clustering of the TOP500 genes was performed by Cluster 3.0 using centroid linkage method. Each row denotes a gene and each column corresponds to a case of ULMS. Red, over-expressed genes; Green, down-expressed genes. (TIFF 2173 kb)

Abbreviations

CDK6: Cyclin-dependent kinase 6; EMT: Epithelial to mesenchymal transition; GSEA: Gene Set Enrichment Analysis; HOXA1: Homeobox A1; IHC: Immunohistochemistry; LMOD1: Leiomodin-1; LMS: Leiomyosarcoma; MAP K13: Mitogen-activated protein kinase 13; MYH11: Myosin-11; MYLK: Myosin light chain kinase; MYOCD: Myocardin; SAM: Significance Analysis of Microarrays; SLMAP: Sarcolemmal membrane-associated protein; SRF: Serum response factor; TCGA: The Cancer Genome Atlas; ULMS: Uterine leiomyosarcoma

Availability of data and materials
The Level 3 RNAseq expression data of uterine leimyosarcoma cases were obtained from The Cancer Genome Atlas (TCGA) database.

Authors' contributions
Conceived and designed the experiments: XG, YA, SJ, WC; Performed the bioinformatic analysis: XG, YA, SW, HW; Performed the statistical analyses: SL, LZ, DW; Performed the data collection: HW; Interpreted the data: XG, YA, SW, HW, DW, WZ, SL, LZ, SZ, YL, WC, JS; Drafted the manuscript: XG, YA, SW; Revised the manuscript: HW, DW, SL, LZ, SZ, WZ, YL, WC, SJ. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
Paraffin blocks of uterine leiomyosarcomas were obtained with Health Insurance Portability and Accountability Act (HIPAA)-compliant Stanford University Medical Center Institutional Review Board approval and a waiver of consent due to the archival nature of the specimens.

Consent for publication
Not applicable

Competing interests
The authors confirm that there are no competing interests.

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Author details
[1]Department of Biochemistry and Molecular Biology, Joint National Laboratory for Antibody Drug Engineering, Institute of Biomedical Informatics, Medical School, Henan University, Kaifeng 475004, China. [2]Cell signal transduction Laboratory, Henan University, Kaifeng 475004, China. [3]Department of Neurology, The First Affiliated Hospital of Henan University, Kaifeng 475001, China. [4]Department of Nuclear Medicine, The First Affiliated Hospital of Henan University, Kaifeng 475001, China. [5]Department of Orthopedic Surgery, Peking Union Medical College, Chinese Academy of Medical Science, Beijing 100730, China. [6]Department of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco, CA 94110, USA. [7]Department of Preventive Medicine, Medical School, Henan University, Kaifeng 475004, China. [8]Institute of Environmental Medicine, Henan University, Kaifeng 475004, China.

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