Strong Smooth Muscle Differentiation Is Dependent on Myocardin Gene Amplification in Most Human Retropertitoneal Leiomyosarcomas

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Abstract

Myocardin (MYOCD), a serum response factor (SRF) transcriptional cofactor, is essential for cardiac and smooth muscle development and differentiation. We show here by array-based comparative genomic hybridization, fluorescence in situ hybridization, and expression analysis approaches that MYOCD gene is highly amplified and overexpressed in human retropertitoneal leiomyosarcomas (LMS), a very aggressive well-differentiated tumor. MYOCD inactivation by shRNA in a human LMS cell line with MYOCD locus amplification leads to a dramatic decrease of smooth muscle differentiation and strongly reduces cell migration. Moreover, forced MYOCD expression in three undifferentiated sarcoma cell lines and in one liposarcoma cell line confers a strong smooth muscle differentiation phenotype and increased migration abilities. Collectively, these results show that human retropertitoneal LMS differentiation is dependent on MYOCD amplification/expression, suggesting that in these well-differentiated LMS, differentiation could be a consequence of an acquired genomic alteration. In this hypothesis, these tumors would not necessarily derive from cells initially committed to smooth muscle differentiation. These data also provide new insights on the cellular origin of these sarcomas and on the complex connections between oncogenesis and differentiation in mesenchymal tumors, [Cancer Res 2009;69(6):2269–78]

Introduction

Malignant soft-tissue sarcomas are a heterogeneous group of mesenchymal cancers accounting for ~1% of all malignant tumors in adult. These tumors are classified by pathologists according to their eventual line of differentiation. Among the well-differentiated sarcomas, tumors exhibiting a smooth muscle differentiation are classified as leiomyosarcomas (LMS) and correspond to 10% to 15% of soft-tissue sarcomas. They are generally localized to the retroperitoneum and less frequently to the limbs (1). These tumors have a poor prognosis and present very rearranged karyotypes, close to that observed in most undifferentiated pleomorphic sarcomas (2). These last undifferentiated sarcomas, which account for 5% of soft-tissue sarcomas (3), are preponderantly observed in limbs and are associated with a slightly better prognosis (1). Genetic similarities and a tendency to display an overlapping immunohistochemical phenotype suggest that both groups of tumors share common oncogenic pathways. Some genomic alterations are frequently observed in both groups, like deletion of chromosome 13, targeting the RB1 gene (4), and deletion of 10q (5, 6), whereas 17p amplification is more specific of LMS. Here, we show that a target of this amplification likely corresponds to the gene encoding MYOCD, a transcriptional cofactor of serum response factor (SRF) regulating smooth muscle development and differentiation (7–9). Moreover, we show that MYOCD expression in sarcoma cell lines not only induces smooth muscle differentiation but also promotes cell migration, a feature that could be mediated by LPP induction. These results suggest that in these well-differentiated LMS, differentiation could be a consequence of an acquired genomic alteration, and that these tumors are not necessarily derived from cells initially committed to smooth muscle differentiation.

Materials and Methods

Tumor samples. Frozen samples and four fresh tumors were obtained from different pathology laboratories. All histologic slides were reexamined by the French Sarcoma Group: 19 tumors were classified as well-differentiated LMS and 17 tumors were classified as pleomorphic undifferentiated sarcomas. According to French law at the time of the study, the tumor samples were collected in agreement with the ANAES (Agence Nationale d'Accréditation et d'Evaluation en Santé) recommendations. Experiments were done in agreement with the Bioethics Law no. 2004-800 and the Ethics Charter from the National Institute of Cancer (INCa).

Cell line establishment and culture conditions. Sterile fragments from resected tumors LMS148, S100, S108, and S137 were minced in culture medium. Experiments were done in agreement with the Bioethics Law no. 2004-800 and the Ethics Charter from the National Institute of Cancer (INCa).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Accession number: The Affymetrix data observed on the 32 human tumors and on the LMS148L cell line have been deposited in the EMBL-EBI database (http://www.ebi.ac.uk/) and are accessible through accession no. E-MEXP-1922.

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100 ng/mL. LMS148L cells cultured in the absence of PDGF-BB were used as control. Smooth muscle–related gene expression was then assessed by real-time PCR from three independent experiments.

**Immunohistochemistry.** Immunohistochemistry was done on paraffin blocks according to the method described previously (2). For cell lines, cells were seeded at a density of 10^6 per well onto two-well Lab-Tek slides (Nunc). Twenty-four hours later, cells were fixed with cold acetone solution for 10 min. After blocking with 2% FCS (Abcam), 2% bovine serum albumin (Sigma) in Tris buffer solution for 30 min, cells were incubated with the primary antibody and then treated in the same manner as the paraffin sections. Experiments were realized with the same antibodies used for Western blot except for α-smooth muscle actin (SMA; M0851, DAKO). Anti-calponin and anti-transgelin antibodies were used at a dilution of 1:1,000; anti-SMA antibody and anti-caldesmon antibodies were used at a dilution of 1:50.

**Human array-based comparative genomic hybridization and image analysis.** Genomic DNA was isolated using a standard phenol-chloroform extraction protocol. Array-based comparative genomic hybridization (array-CGH) experiments were done with a DNA microarray developed in our laboratory. Three thousand eight hundred seventy-four BAC/PAC DNAs (BACPAC Resources Center, Children's Hospital Oakland Research Institute) were spotted in triplicate on Ultragaps slides (Corning). These clones cover up the whole genome with a resolution of 1 Mb. The probes were prepared and hybridized as previously described (10). The data were analyzed by a software developed at Institut Curie.6 Cyanine-5/cyanine-3 ratios >2 were considered as amplifications, and ratios >1.2 and <0.8 were considered as gains and losses, respectively.

**Fluorescence in situ hybridization.** Metaphase spreads were obtained by standard procedures. For frozen tissues, 5-μm-thick tumor sections were used. Probes used refer to BAC RP11-746E8, which overlaps the MYOCD genomic sequence (located in band 17p12), and BAC RP11-388C12, located in band 17q25.3, as a control probe. Fluorescence in situ hybridization (FISH) was done as previously described (10). Photographs were obtained using a Zeiss Axioplan 2 microscope, a Photometrics Quantix CCD camera, and the SmartCapture v2 Digital Scientific software.

**Transcriptome analysis.** Total RNA was extracted from frozen tumor samples or from cell line with TRIzol reagent (Life Technologies). RNA was then purified using the RNeasy Min Elute Cleanup Kit (Qiagen) according to manufacturer's procedures. RNA quality was checked on an Agilent 2100 bioanalyzer (Agilent Technologies). Samples were then analyzed on Human Genome U133 Plus 2.0 array (Affymetrix) according to the manufacturer's procedure. All microarray data were simultaneously normalized using the GCIRMA algorithm (11). Hierarchical and biological variance were done using the dChip software (average linkage method, rank correlation distance metric). For more details, see Supplementary Methods.

**Western blot.** Total proteins of tumors were extracted by crushing frozen tumor samples in 8 mol/L urea (Interchim Upima). Radioimmunoprecipitation assay buffer is used for cell line total proteins extraction. The primary antibodies and dilutions used in this study are as follows: antibodies to MYOCD (1:200; A-13, Santa Cruz Biotechnology), CNNI (1:10,000; C2687, Sigma-Aldrich), SM22a (TAGLN; 1:10,000; ab14106, Abcam), ICALD (1:1,000; M5537, DAKO), α-SMA (1:400; A2547, Sigma-Aldrich), GKLK (KLF4; 1:500; H-180, Santa Cruz Biotechnology), PDGF (1:100; Ab-1, 101-116, Calbiochem), LPP (1:500; Ab63621, Abcam), hemagglutinin (HA; 1:10,000; 12CA5, Roche), or β-actin (1:1,000; Sigma-Aldrich). Secondary antibodies were horseradish peroxidase conjugated: antirabbit IgG (1:3,000; NA934, Amersham), antimouse IgG (1:3,000; NA931V, Amersham), or antigoat IgG (1:1,000; sc-2020, Santa Cruz Biotechnology). Western blot images were acquired with a Fujifilm luminense image LAS-1000plus analyzer. They were analyzed with Image Gauge V4.0 software, which allows us to normalize protein of interest expression data to β-actin expression values.

**siRNA.** SIGENOME SMARTpool siRNA (Dharmacon) directed against myocardin (MYOCD) and siCONTROL Non-Targeting siRNA pool (Dharmacon) were used to perform MYOCD expression knockdown experiments in LMS148L. Negative Control siRNA Alexa Fluor 488 (Qiagen) and siControl Human Cytosplalin B siRNA (Dharmacon) were used as transfection controls. LMS148L cells were seeded at a density of 1.2 × 10^5 per well onto six-well plates 24 h before transfection. The cells were washed with Opti-MEM (Life Technologies) and then incubated for 5 h at 37°C in 60 μL of Opti-MEM and 15 μL of Oligofectamine reagent (Invitrogen) containing 100 pmol of the siRNA of interest. The same steps were reproduced for the second transfection at 3 d. Each transfection was done twice for all siRNAs. For real-time PCR experiments, gene expressions in cell lines transfected with MYOCD specific siRNA were compared with those in cells in lines transfected with the nontargeting siRNA control. A mean of inhibition percentages was then calculated for each time point for each gene.

**shRNA.** RNAi retroviral shRNA (RHS6001) with three shRNA mir directed against MYOCD (shRNA1.14: RHS1764-9697831; shRNA1.8: RHS1764-9403778 and shRNA1.3: RHS1764-9100135) were used as transfection controls. LMS148L cells were seeded at a density of 2 × 10^4 per well onto eight-well Lab-Tek slides (Nunc). Twenty-four hours later, cells were fixed with a 3.7% paraformaldehyde solution for 20 min. After washing, the filter were removed by wiping with cotton swabs, and the cells on the lower surface were photographed (Coolpix 8400, Nikon) under a microscope (Eclipse TS100, Nikon).

**Immunofluorescence.** Cells were seeded in duplicate at a density of 10^4 per well onto eight-well Lab-Tek slides (Nunc). Twenty-four hours later, cells were fixed with a 3.7% paraformaldehyde solution for 20 min. They were then permeabilized with 0.1% SDS in PBS 1× for 10 min. After each treatment step, cells were washed with PBS 1×. After blocking with 10% FCS in PBS 1× for 20 min, cells were incubated with anti-TAGLN or anti-CNN1 antibodies at a dilution of 1:500 and 1:1,000, respectively, in PBS 1× with 10% FCS for 1 h at room temperature. Then, cells were incubated with rabbit immunoglobulins/TRITC or mouse immunoglobulins/FITC (DAKO) at a dilution of 1:50 and 1:400, respectively, in PBS 1× with 10% FCS for 1 h at room temperature. Immunofluorescence experiments were done with the same antibodies used for Western blot, and photographs obtained as described above in FISH section.

**Migration assays.** The cell migration activity was assayed in Boyden chambers with polycarbonate membrane of 8.0-μm pore size (Corning). Cells were suspended to a final concentration of 5 × 10^4/mL in medium with 0.5% FCS and were added to the upper compartment. Medium, with 0.5% FCS or 10% FCS as a chemoattractant, was added to the plate well and cells were incubated for 24 h at 37°C. The cells on the upper surface of the filter were removed by wiping with cotton swabs, and the cells on the lower surface were stained with crystal violet. The cells that had invaded to the lower surface areas were photographed (Coolpix 8400, Nikon) under a microscope (Eclipse TS100, Nikon).

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6 http://bioinfo3.curie.fr/HumanCGHarray/
Figure 1. Genomic amplification of the 17p11.2-17p12 region. A, CGH results on 28 BAC-PAC clones covering the major part of 17p chromosome. The names of these clones and their genomic locations are indicated on the left. The names of studied tumors are indicated at the top, and retroperitoneal tumors are shaded in gray. The genomic status (tumor DNA/normal DNA ratio) of each tumor for each locus is indicated by a color code in filled squares, as defined at the bottom. Position of MYOCD gene is indicated as well as the minimal common region of amplification (open black box).

B, FISH on frozen sections of tumors LMS104, LMS29, and LMS79 and on metaphase spreads from the LMS148L cell line. The MYOCD probe corresponds to BAC RP11-746E8 and is labeled with Cy3-dUTP (yellow). The control locus, corresponding to the telomeric clone RP11-388C12 on the long arm of chromosome 17, is labeled with Cy5-dUTP (pink). Genomic amplification of this locus is observed in all samples. Inset, the MYOCD gain in the LMS148L cell line is enlarged. Bar, 10 µm.
For measurement of cell migration by wound healing assay, confluent cells were wounded with a plastic micropipette tip having large orifice. The experiment was done twice in duplicate for each cell line. Photographs of three randomly selected points along each wounded area were taken just after wounding and after 24, 32, and 48 h (Coolpix 8400, Eclipse TS100, Nikon).

**Lentiviral constructs and infections.** For lentiviral expression, HA-tagged MYOCD-encoding sequence obtained from GeneCopoeia (EX-E0450-Lv06) was amplified by PCR with added 5′-end Xba1 and 3′-end Swa1 restriction enzyme sites and then was cloned between these restriction sites in the lentiviral pCDH1 vector (SBI). Viral particles were produced in HEK-293T cells transfected with appropriate packaging plasmids by the calcium phosphate method. Viral supernatants were supplemented with 8 μg/mL polybrene for infection and puromycin was added 24 h later.

**Results**

**Tumor samples.** A series of 37 samples were analyzed (36 frozen tumors and 1 cell line). Their main clinical and pathologic data are indicated in Supplementary Table S1. These tumors were extensively characterized by immunohistochemistry (Supplementary Fig. S1). Tumors were classified by pathologists of the French Sarcoma Group as well-differentiated LMS according to their morphologic features and when they exhibit a positive immunolabeling for all smooth muscle–related genes. As shown in Supplementary Table S1, our series of frozen tumors consists of 19 well-differentiated LMS and 17 pleomorphic undifferentiated or poorly differentiated sarcomas. All these tumors were analyzed by array-CGH and they all present complex rearranged karyotypes. Transcriptomes of 16 of 19 LMS and 16 of 17 undifferentiated sarcoma samples were analyzed using U133Plus 2.0 Affymetrix arrays. For this purpose, data were normalized using the GCRMA algorithm.

**Most well-differentiated LMS present an amplification of the 17p11.2-p12 genomic region.** In the present sarcoma series, increased copy number or amplification were recurrently observed...
Figure 3. MYOCD overexpression in amplified LMS. A, MYOCD and SRF expression levels estimated by real-time PCR. The tumor groups are indicated: group A, LMS with 17p amplification; group B, LMS without 17p amplification; group C, undifferentiated sarcomas with 17p gain; group D, undifferentiated sarcomas without 17p gain. Boxplots corresponding to each bar chart are shown on the right. B, relative expression level of MYOCD estimated by real-time PCR correlates with the genomic amplification level obtained by array-CGH experiments (Pearson’s correlation). C, boxplot analysis of MYOCD protein expression between the different tumor groups (P value using Student’s t test). Bottom right, a representative Western blot with anti-MYOCD antibody.
by array-CGH for clones corresponding to 17p11.2-17p12 loci (Fig. 1A), with a minimal common region of gain at coordinates chr17:12,436,554–17,743,261 in the Human March 2006 Assembly at UCSC. This overrepresentation of 17p chromosome was essentially observed in LMS (10 LMS among 16 amplified tumors) and particularly in those with retroperitoneal occurrence: 8 of 11 retroperitoneal LMS do present this amplification, whereas only 2 of 8 non-retroperitoneal LMS have the 17p amplification. Moreover,
high-level amplification of 17p chromosome (array-CGH ratio > 2) was exclusively observed in LMS. Genomic amplification was confirmed by fluorescence in situ hybridization (FISH) on frozen tissue sections from 8 tumors and on metaphase spreads from the LMS148L cell line, with a probe corresponding to the most amplified locus (clone RP11-746E8). High copy number of this locus was confirmed in all tumors (Fig. 1B) when compared with a 17q control probe (RP11-388C12 BAC clone). In metaphase from LMS148L, a cell line established from a LMS, a complex rearrangement was observed on a derivative chromosome 17 with a gain of the RP11-746E8 locus (Fig. 1B).

**MYOCD gene is the most overexpressed gene in the minimal amplified region.** An unsupervised hierarchical clustering was done with dChip software using normalized transcriptome data. As shown in Fig. 2A, two main tumor clusters corresponding to the well-differentiated LMS on one hand and to the undifferentiated sarcomas on the other hand were observed. Each of these two clusters is subdivided, and for example, in the LMS cluster, most of the amplified tumors are grouped together. In a first step, we analyzed the relative expression of genes from the major part of the 17p chromosome between LMS with amplification and undifferentiated sarcomas. As shown in Fig. 2B, two probe sets exhibit a dramatic increase in relative expression: Both correspond to a gene that encodes the MYOCD protein, a SRF transcription cofactor regulating smooth muscle differentiation (7–8, 14). Moreover, this gene is contained in clone RP11-746E8 and thus located in the region with higher amplification level. These results strongly suggest that this gene could be a major target of the ampiclon. In a second step, we performed a Welch test to determine the genes that are differentially expressed between LMS and undifferentiated sarcomas. The MYOCD gene is detected among the top 100 genes and is the first from the amplified 17p region, a result which strengthens the previous hypothesis. When we compared by Welch test LMS with amplification and undifferentiated sarcomas, MYOCD gene is the third most significantly differentially expressed gene (and the first from the 17p ampiclon). Finally, we performed a supervised analysis by between-group analysis (15) of LMS with amplification and undifferentiated sarcomas. As shown in Supplementary Fig. S3A, five of them exhibit a high expression level of KLF4 (Supplementary Fig. S3B), a gene recently described as a smooth muscle differentiation inhibitor (16), both by down-regulating MYOCD expression and by interfering with SRF/MYOCD complex to prevent its association with smooth muscle–related gene promoters. In the same manner, S81 and S80 express high level of PDGFB (Supplementary Fig. S3B), which is implicated in the same differentiation repressive pathway (16, 17). In accordance with this hypothesis, treatment of the LMS148L cell line with human recombinant PDGF-BB leads to a strong decrease of MYOCD gene expression and of the smooth muscle–related genes (Supplementary Fig. S3C).

**In vitro MYOCD inactivation leads to a decrease of smooth muscle differentiation.** It has been well demonstrated that MYOCD forced expression in different cellular models induces smooth muscle–related gene expression (7, 8, 18–21). The strong correlation between MYOCD gene expression and its...
amplification, observed in most well-differentiated LMS, fits well with these data. To confirm the involvement of MYOCD amplification in LMS differentiation, we first transitorily inhibited MYOCD in LMS148L using specific SMARTpool siRNAs. As indicated above, this cell line presents a complex rearrangement of 17p11.2-p12 (Fig. 1B), associated with an overrepresentation of the MYOCD locus and with a strong expression of the MYOCD gene together with the other smooth muscle–related genes (Fig. 3; Supplementary Fig. S2A and B). In this siRNA experiment, a strong inhibition of MYOCD was obtained, associated with a parallel dramatic decrease of smooth muscle–related gene expression, particularly at day 4 when the inhibition of MYOCD is maximal (Supplementary Fig. S4). To more precisely document the link between MYOCD expression and smooth muscle differentiation, we established stable LMS148L cell lines transfected with MYOCD specific shRNAs (sh1.14 and sh1.8) and with a nontargeting shRNA (NT). We then isolated two different subclones from LMS148L transfected with shRNA1.14 (sh1.14C1 and sh1.14C2). As shown in Fig. 4A and B, an important inhibition of MYOCD gene and smooth muscle–related proteins was observed in the sh1.8 and sh1.14 cell lines and in the sh1.14C1 and sh1.14C2 subclones. These data were confirmed by immunocytochemistry (Fig. 4C): Indeed, in shRNA cell lines, a dramatic disorganization of the smooth muscular fibers is observed, with a perinuclear relocalization of the remaining TAGLN and CNN1 proteins. All these results strongly support the lead role of MYOCD gene amplification in producing the features of smooth muscle differentiation observed in LMS.

MYOCD inhibition leads to a decrease of cell migration. MYOCD family members have been described as potential regulators of cell migration (9). To document if MYOCD expression could modulate migration in a LMS context, we performed a series of migration assays for 24 hours in Boyden chambers on shRNA-treated cell lines. As shown in Fig. 5 and Supplementary Fig. S5, shRNA control cells (shNT) were observed in the lower chamber. This migration was almost completely abolished in MYOCD shRNA cell lines (sh1.8, sh1.14, sh1.14C1, and sh1.14C2). We then performed a wound healing experiment on the same cell lines (Supplementary Fig. S6). In LMS148L and in control cells, the wound was healed at 24 h, and the cells were confluent at 32 hours. In contrast, the MYOCD shRNA cell lines did not show wound healing at 32 hours and were not completely confluent at 48 hours.

It has been recently shown that LPP promotes smooth muscle cell migration (22, 23) and that its expression is regulated by MYOCD (22, 24). Indeed in our series, LPP is overexpressed in LMS compared with undifferentiated sarcomas (Supplementary Fig. S7), and its expression is decreased when MYOCD expression is inhibited by shRNAs in LMS148L (Fig. 4B). These results suggest...
that, in a LMS context, increased migration could be due to the up-regulation of LPP expression by MYOCD overexpression.

**MYOCD forced expression in undifferentiated sarcoma cell lines leads to a robust smooth muscle differentiation and increases cell migration.** We then performed forced MYOCD expression experiments, using a lentiviral vector, in three undifferentiated sarcoma cell lines (S100L, S108L, and S137L) and in a liposarcoma cell line (LPS449). As shown in Fig. 6A and Supplementary Fig. S8, forced HA-tagged MYOCD expression was obtained, leading to a strong smooth muscle differentiation in all cell lines, as previously observed in different cellular models (7, 8, 18–21).

We also show that MYOCD up-regulation in sarcoma cell lines strongly promotes migration (Fig. 6B) and LPP expression (Supplementary Fig. S8). These results fit well with those described above after MYOCD inhibition by shRNAs.

**Discussion**

It is generally accepted that most oncogenic processes are associated with a progressive loss of the cell differentiation status, conferring a more proliferative and aggressive phenotype (25–31). Nevertheless, LMS have a very poor prognosis, in spite of a strong differentiation, suggesting that in this cellular context, differentiation and proliferation are not necessarily antinomic. This peculiar situation could be favored by the fact that smooth muscle cells do not reach a terminal differentiation but retain a remarkable plasticity, converting from a quiescent well-differentiated to a highly proliferative state. In some physiologic instances, smooth muscle cells may even grow in a maintained differentiated contractile phenotype (32–34).

Rearrangements of 17p chromosome region have been described in constitutional disorders (35, 36) or in malignancies (37–39), especially in LMS (2, 40). The present analysis not only confirms the occurrence of a highly recurrent 17p11.2-17p12 amplification in LMS but also shows that this amplification is predominantly observed in tumors located at the retroperitoneum. As previously proposed (41), these data suggest not only that this region contains key genes involved in LMS biology but also that the genetics of LMS could be linked to the localization of the tumor.

Our approach, combining array-CGH and transcriptome analysis, shows that the MYOCD gene is localized in the region with the highest amplification level, and that it is the most differentially expressed gene between LMS with the 17p amplification and undifferentiated sarcomas. Furthermore, its expression is hugely correlated with its level of genomic amplification. MYOCD protein, a SRF transcription cofactor, regulates smooth muscle differentiation (7, 8, 14). Its inactivation by siRNA and shRNAs in the LMS148L cell line leads to a dramatic decrease of smooth muscle-related genes. As previously proposed (45), these tumors could be of vascular origin, with a constitutive expression of the MYOCD and MRTF genes, or from cells carrying a more subtle alteration leading to an indirect activation of these genes.

Finally, we show that MYOCD expression is strongly associated with the migration ability of the cells, whatever the cellular context. This feature could be due to the up-regulation of LPP, a MYOCD target involved in smooth muscle cell migration (22–24). MYOCD overexpression could thus be involved in the particularly high rate of metastases observed in LMS.

In conclusion, our data show that a chromosomal rearrangement acquired during oncogenesis, could both compel tumor cells to reach a strong specific differentiation and participate in the metastasis process.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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