**Let-7 repression leads to HMGA2 overexpression in uterine leiomyosarcoma**

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**Abstract**

Overexpression of HMGA2 is common in uterine leiomyomas (ULM). The expression of HMGA2 in its malignant counterpart – uterine leiomyosarcomas (ULMS) remains undetermined. Recently it has been shown that repression of HMGA2 by microRNA let-7s is a critical molecular regulatory mechanism associated with tumour growth in many tumours and cell types, including leiomyomas. To test whether HMGA2 and let-7s play a role in ULMS, we examined the levels of endogenous HMGA2 and let-7 expression and found a significant correlation between these two molecules in a case-matched cohort of human ULMS. We found that overexpression of HMGA2 and let-7-mediated HMGA2 repression is a relevant molecular alteration in ULMS. Disrupting the control of HMGA2 and let-7 pairs promotes ULMS cell growth in vitro.

**Introduction**

Uterine leiomyosarcomas (ULMS) are rare neoplasms, representing 1 in 200 to 800 smooth muscle tumours or ~1% of all malignancies of the uterus [1]. Although the reported 5-year ULMS survival rates are variable, these tumours are clinically aggressive and have a high risk of recurrence and overall poor prognosis [2]. The cause or the molecular basis of human ULMS is largely unknown. While it was believed that most ULMS could arise de novo, some reports suggest that ULMS was progressive from pre-existing uterine leiomyomas (ULM) [3, 4]. However, the conclusive evidence documenting the transformation of ULM to ULMS is lacking in human beings and it is thought that the incidence of transformation is <0.1% [5].

HMGA2, a high-mobility-group AT-hook (HMGA) protein, is considered to function as an oncogene strongly associated with many malignant epithelial and mesenchymal neoplasms [6]. It has three AT-hook DNA binding domains, through which HMGA2 binds to AT-rich sequences in the minor groove of the DNA helix. HMGA2 is expressed in embryonic tissue but not in most adult tissues [7, 8]. HMGA2 is overexpressed in ULM due to chromosomal 12q15 changes [9–12]. Overexpression of HMGA2 in ULMS has been suggested, but not fully investigated [4, 13]. HMGA2 is an important regulator of cell growth, differentiation, apoptosis and transformation [14]. New evidence suggest that dysregulation of microRNAs (miRNAs) may play a central role in tumour development [15]. Members of the let-7 miRNA family function as tumour suppressors through specific repression of its target gene, particularly of HMGA2 expression in some tumour cells both in vivo and in vitro [4, 16, 17]. The biological importance of molecular pairing of HMGA2::let-7 was further illustrated by the demonstration that repression of HMGA2 by let-7s impairs tumour cell proliferation in many different tumour types, including ULM [4, 16–19]. It would be of great interest to characterize whether overexpression of HMGA2 and disruption of the HMGA2::let-7 pairs contributes to the aggressive growth behaviour of ULMS.

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In this study, we examined the levels of endogenous HMGA2 and let-7 expression and analysed a potential correlation between these two molecules in a case-matched cohort of human ULMS. We demonstrate that overexpression of HMGA2 and let-7-mediated HMGA2 repression is important for molecular changes in ULMS. Disrupting the HMGA2::let-7 pairs promote ULMS cell growth in vitro.

Materials and methods

Patient and tissue samples

A total of 35 hysterectomies for ULMS were collected in this study. The mean ages at hysterectomy were 59.9 years old (ranging from 38 to 83 years old). Tumour sizes were ranged from 2 to 22 cm with mean tumour size of 11.6 cm. Of all 35 cases, 23 were solitary and 12 coexisted with leiomyomas (Table 1).

High-density tissue microarray (TMA) was prepared from formalin fixed and paraffin-embedded (FFPE) tissue cores (0.6 mm) in ULMS (n = 30) and matched myometrium (n = 30) in triplicate. Fresh frozen tissues from ULMS and matched myometrium were collected in five cases for RT-PCR and Western blot analysis (Table 1).

Three uterine leiomyosarcoma cell lines were used for the study including: SK-LMS-1, SK-UT-1 and SK-UT-1b.

The study was approved by the NYU Medical Center institutional review board.

Let-7 mimic and inhibitor

Mature double stranded miRNAs of let-7 and let-7 inhibitors were purchased from Dharmacon, Inc. (Lafayette, CO, USA). All experiments were controlled using a non-functional double-stranded random 22 nt RNA (Block-iT, Invitrogen, Carlsbad, CA, USA).

Primers and antibodies

Primers from HMGA2 and its alternative spliced transcripts were reported previously [19]. Primers for mature let-7 family (a–i) were purchased from Ambion, Inc. (Austin, TX, USA). Antibodies includes HMGA2 (provided by Dr. Masashi Norita and Biocheck, CA, USA), Ki-67, ERα and PR-A (Ventana Medical System, Inc., Tucson, AZ, USA), and P53 (Neomarkers, CA, USA).

Let-7c miRNAs in situ hybridization

The hybridization system and probes, miRCURY LNA, Let-7c, and U6, were purchased from Exiqon (Vedbaek, Denmark). The detailed procedure for in situ hybridization was followed as per manufacturer’s protocol [20]. In brief, 4-μm TMA slides were prepared. Following deparaffinization and deproteinization, the slides were pre-hybridized with 1× hybridization buffer without probe. The hybridization was carried out overnight in a 1× hybridization buffer (30–70 μl) with pre-denatured miRCURY LNA, let-7c or U6 probes. After washing, the slides were blocked and incubated with AP conjugated anti-DIG Fab fragments (1:1500, Roche, Indianapolis, IN, USA) and visualized for colour detection.

qRT-PCR

For the detection of mature miRNAs, mirVana qRT-PCR primers and the mirVana qRT-PCR Detection Kits (Ambion, Inc.) were used and optimized according to the abundance of miRNAs in the samples. A total of 15–30 cycles were performed for quantitation. Primers for the common domain of HMGA2, the dominant transcript (HMGA2a), and the cryptic HMGA2 transcripts were described [19]. The abundances of cDNA products were detected by qRT-PCR and were normalized by the internal control products of U6 and α-Actin.

Immunohistochemistry

The TMA blocks from FFPE tissues were sectioned at 4 microns. After deparaffinization and antigen retrieval, all immunohistochemical staining was performed on a Ventana Nexus automated system.

Western blot analysis

Fresh frozen tissue or culture cell samples were homogenized at 4°C in a protein lysis buffer (0.5 g tissue in 1–2 ml). Identical amounts of total proteins from each sample were separated through a 12% SDS-PAGE gel and then transferred to a PVDF membrane (Perkin Elmer Life Scientific Inc.). Development of the immunoblot with antisera against HMGA2 and negative control HMGA2 blocking peptide (provided by Dr. Masashi Norita and Santa Cruz Biotechnology, Inc., CA, USA) was tested and a single specific HMGA2 band at 25 kD was detected, as previously described.

Cell culture and miRNAs transfection

LMS cell lines were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% foetal bovine serum (Gemini, Calabasas, CA, USA) in 37°C incubators with 5% CO2 until the cells reached 30–40% confluence. Prior to transfection, cells were placed in standard media without antibiotics for 24 hrs. As per manufacturers’ protocol, transfection was performed with the Lipofectamine system with miRNAs concentrations of 20–60 pmol/well in either 6- or 24-well plates. To estimate transfection efficiency, cotransfection with the block-iT fluorescent double-stranded random 22mer RNA from Invitrogen was performed. The FITC fluorescence was visualized by λex = 494 nm and λem = 519 nm to assess the percentage of cells that were successfully transfected. Cells receiving only the tagged random sequence double-strand 22mer were used as non-specific references at all data points. Following transfection, cells were harvested and analysed at the indicated times.

Cellular proliferation assay

PC3 and LNCaP cell lines of control (with pBabe) and of tests (stable over-expression of HMGA2a with and without let-7 LCS) were passed in 24-well plates in triplicate at densities of 5 × 103 cells/well for LNCaP and 1 × 104 cells/well for PC3.
### Table 1  HMGA2 and let-7s expression in 35 uterine leiomyosarcomas

| Case no. | Age (years) | Size (cm) | HMGA2 (IHC) | HMGA2 (RT) | Let-7c (ISH) | Let-7 (c, d, f-2) | Ki-67 index (%) |
|----------|-------------|-----------|-------------|------------|--------------|------------------|-----------------|
| ULMS1    | 43          | 5         | 0           | 0          | 30           |                  |                 |
| ULMS2    | 57          | 4         | 0           | 0         |              |                  | 15              |
| ULMS3    | 55          | 4         | 0           | 0         |              |                  | 50              |
| ULMS4    | 53          | 10.5      | 0           | 0         |              |                  | 60              |
| ULMS5    | 54          | 11        | 0           | 0         |              |                  | 50              |
| ULMS6    | 79          | 30        | 0           | 0         |              |                  | 25              |
| ULMS7    | 83          | 13        | 2+          | 0         |              |                  | 25              |
| ULMS8    | 38          | 17        | 0           | 0         | 15           |                  | 25              |
| ULMS9    | 82          | 9         | 0           | 0         |              |                  | 35              |
| ULMS10   | 69          | 8.5       | 3+          | 1         |              |                  | 25              |
| ULMS11   | 85          | ?***       | 3+          | 0         |              |                  | 25              |
| ULMS12   | 59          | 15        | 0           | 0         |              |                  | 30              |
| ULMS13   | 74          | 5         | 0           | 0         |              |                  | 5               |
| ULMS14   | 50          | 6.8       | 0           | 0         |              |                  | 50              |
| ULMS15   | 52          | 22        | 3+          | 0         |              |                  | 50              |
| ULMS16   | 60          | 12        | 2+          | 0         |              |                  | 40              |
| ULMS17   | 55          | 9         | 0           | 0         |              |                  | 15              |
| ULMS18   | 73          | 11        | 2+          | 0         |              |                  | 15              |
| ULMS19   | 46          | ?          | 0           | 0         |              |                  | 20              |
| ULMS20   | 64          | ?          | 3+          | 0         |              |                  | 35              |
| ULMS21   | 64          | 8         | 0           | 0         |              |                  | 30              |
| ULMS22   | 48          | 7         | 0           | 0         |              |                  | 5               |
| ULMS23   | 63          | ?          | 0           | 0         |              |                  | 5               |
| ULMS24   | 59          | 4.5       | 0           | 0         |              |                  | 60              |
| ULMS25   | 55          | ?          | 0           | 0         |              |                  | 60              |
| ULMS26   | 43          | 20        | 3+          | 0         |              |                  | 20              |
| ULMS27   | 57          | 12        | 4+          | 0         |              |                  | 30              |
| ULMS28   | 49          | 14        | 3+          | 0         |              |                  | 50              |
| ULMS29   | 74          | ?          | 3+          | 0         |              |                  | 40              |
| ULMS30   | 60          | 2         | 0           | 0         |              |                  |                 |
| ULMS31   | 55          | 16        | 3           | 0         |              |                  |                 |
| ULMS32   | 63          | 17        | 2*          | 2         |              |                  |                 |
| ULMS33   | 51          | 20        | 3           | 3         |              |                  |                 |
| ULMS34   | 53          | 21**       | 2*          | 2         |              |                  |                 |

Continued
for PC3 cells. Cells were subsequently transfected with control RNA (non-function, Invitrogen), let-7c, and/or let-7 inhibitors (Dharmacon, Inc.) at a dose of 40 pmol/well. Cellular proliferation was counted at 24, 48, 72 and 96 hrs using the colorimetric WST-1 assay (Cell proliferation Reagent, Roche). Briefly, the cells were incubated with 10% WST-1 reagent in normal medium for 2 hrs. Aliquots (100 μl) were then transferred to 96-well plates and the samples were read in a spectrophotometric plate reader at 450 nm (FLUOstar OPTIMA, BMG Lab Technologies, Durham, NC, USA).

Statistical analysis

Mean and standard errors were calculated for the quantitative values. Statistical significance was analysed by a paired t-test and P-value <0.05 was considered significant.

Results

HMGA2 and let-7 expression in ULMS

To evaluate whether elevated HMGA2 expression is common in ULMS, we examined HMGA2 expression in 30 ULMS by immunohistochemistry analysis. With internal negative controls of matched myometrium and staining done with two different sources of HMGA2 antibodies (see section ‘Materials and methods’), we found up to 38% of ULMS (11/29) immunoreactive for HMGA2 (Fig. 1). We then examined HMGA2 expression at transcription and translational levels by RT-PCR and Western blot, respectively, in five randomly selected ULMS collected from fresh frozen tissues. With a negative (matched myometrium) and a positive (HMGA2 positive ULM) controls, a moderate to high level of HMGA2 mRNA expression (32 cycles) was detected in all five cases (Fig. 2A). Of same five tumours, two of them were positive for HMGA2 by Western blot analysis (Table 1, Fig. 2A).

The karyotype analysis was carried out in the latter five cases (ULMS31–35). One (ULMS-34) of five ULMS had non-random chromosomal changes involving chromosome 12 (Fig. 2B). These non-random chromosome 12 changes were commonly seen in usual type ULM. Further analysis of this tumour revealed a high level of HMGA2 mRNA and protein (Fig. 2A). This tumour had all the features of malignant uterine smooth muscle tumour, including tumour necrosis, cytological atypia, invasion and high mitotic counts (Fig. 2B). We speculated that overexpression of HMGA2 in ULMS-34 was contributed by chromosome 12 changes same as those seen in usual ULM.

The levels of Let-7 expression in ULMS were low based on semi-quantitative RT-PCR analysis in 5 ULMS (Fig. 2A). Among five let-7 family members (let-7a, b, c, e, f-2) examined, let-7c transcript was detectable in all ULMS. We therefore selected let-7c to analyse its expression in 30 ULMS by TMA based in situ hybridization (see section ‘Materials and methods’). We used U6 as tissue quality control (Fig. 1B). The relative levels of let-7c were first normalized by U6 (let-7c/U6 × 100%), and the net changes of let-7c in ULMS against matched myometrium (ULMS myometrium) were further calculated. Overall, there were slightly net losses of let-7c in ULMS in comparison to matched myometrium (−0.35 ± 0.15). A case-matched analysis of HMGA2 and let-7c expression was performed. As illustrated in Fig. 1A, in those ULMS with positive immunoreactivity for HMGA2, net loss of let-7c expression was quite more common than in tumours negative for HMGA2. Correlation analysis showed an inverse association between HMGA2 and let-7c expression (r = −0.39) (Table 1, Fig. 1A). In those five cases with RT-PCR data, higher levels of HMGA2 mRNA in ULMS-32 and -34 were accompanied by lower levels of let-7s (Fig. 2A).

Correlation analyses were performed between HMGA2 expression and tumour size, tumour markers of Ki-67, P53, ERα and PR-A. We found a weak positive correlation of HMGA2 expression and tumour sizes (r = 0.35). There was not significant correlation of HMGA2 with other immunomarkers.

Translational regulation of HMGA2 by let-7s in ULMS cell lines

To test whether endogenous let-7s play a major role in repression of HMGA2 in ULMS, we selected three ULMS cell lines for the study. We first examined HMGA2 and its cryptic transcript (isiforms) [12, 21] expression in these cell lines by RT-PCR analysis. Since HMGA2a and its cryptic transcripts can be induced in primary
cultured leiomyoma cells [12], we used cultured leiomyoma cells as a positive control. We found all ULMS cell lines had a high abundance of HMGA2a mRNA (Fig. 3A). In addition, HMGA2 cryptic transcripts c, d and f showed moderate levels in these cell lines (Fig. 3A). In contrast, expression of let-7s was quite low in comparison to normal myometrium and ULM based on a semi-quantitative analysis (Fig. 3B). Let-7c and f were the most abundant transcripts of let-7 family in these cell lines.

To test the role of let-7s in repression of HMGA2 after transcription and translation, we transfected the exogenous let-7c mimic.
and let-7 inhibitor in all cell lines. In untreated cell lines (controls), low levels of endogenous let-7s seemed not enough to destabilize HMGA2a and other cryptic mRNA (Fig. 3A and B), but were sufficient to repress HMGA2 translation (Fig. 3D, control lanes). When applying 40 pmol of exogenous let-7c, a significant reduction of HMGA2a, c, and f mRNAs (Fig. 3C) was observed, and a complete repression of HMGA2 protein was evident by a Western blot analysis (Fig. 3D, let-7c channel). When the cells treated with exogenous let-7c and let-7 inhibitor were validated as shown in Fig. 3C and D, we counted for the tumour growth rates at days 1, 2 and 3 in all three ULMS cell lines (Fig. 4). Starting from day 2 (48 hrs), cells treated with let-7c had significantly lower rates of tumour cell growth than control ($P < 0.05$). The differential growth rates were even wider at day 3 (72 hrs). Particularly, the growth rates in tumour cells treated with let-7 inhibitor were significantly higher than those in let-7c treated cells ($P < 0.05$), but at least two cell lines lower than those in control groups (Fig. 4). The later findings suggested that let-7 mediated tumour growth in ULMS is not solely through repression of HMGA2, and other let-7 target genes may be involved [22] (Fig. 4).

**Repression of HMGA2 inhibited ULMS cell growth**

To test whether HMGA2 in ULMS cell lines plays a role in regulation of tumour growth rate, we examined the tumour cell proliferation by introducing exogenous let-7 and let-7 inhibitor, respectively. The cells were treated in triplicate with Block-iT (control), let-7c and let-7 inhibitor. Repression of HMGA2 by let-7c and induction of HMGA2 overexpression by let-7 inhibitor were validated as shown in Fig. 3C and D, we counted for the tumour growth rates at days 1, 2 and 3 in all three ULMS cell lines (Fig. 4). Starting from day 2 (48 hrs), cells treated with let-7c had significantly lower rates of tumour cell growth than control ($P < 0.05$). The differential growth rates were even wider at day 3 (72 hrs). Particularly, the growth rates in tumour cells treated with let-7 inhibitor were significantly higher than those in let-7c treated cells ($P < 0.05$), but at least two cell lines lower than those in control groups (Fig. 4). The later findings suggested that let-7 mediated tumour growth in ULMS is not solely through repression of HMGA2, and other let-7 target genes may be involved [22] (Fig. 4).

**Discussion**

ULMS usually have very complicated cytogenetic alterations involving many different chromosomal regions. Cytogenetic
changes involving chromosomal 12q13–15 region in leiomyosarcomas had been occasionally reported through traditional karyotype [23] and comparative genomic hybridization (CGH) analysis [4], in which a gain of chromosome 12q15 region including HMGA2 was identified. Examination of HMGA2 expression in anatomic sites other than the uterus revealed overexpression of HMGA2 mRNA in 57% of leiomyosarcomas by RT-PCR [13]. Examination of HMGA2 expression in ULMS has not been reported in the literature. This study is the first attempt to examine HMGA2 expression in ULMS. In this study, we observed that over one third of ULMS were immunoreactive for HMGA2. Furthermore, overexpression of HMGA2 mRNA was observed in all native ULMS and ULMS cell lines in a small scale analysis. Given that only one of five ULMS showed chromosomal 12 changes, overexpression of HMGA2 in ULMS seemed to be through an alternative mechanism.

The specific role of HMGA2 in tumourigenesis remains to be determined. Recent characterization of let-7 regulation of HMGA2 indicates that this molecular pair plays a critical role in the control of mitogenic activity in many different cell types and tumour cells [17, 18, 24, 25]. Particularly, it has been found that dysregulation of let-7s is associated with HMGA2 expression in ULM and related to cell proliferation [17]. The later findings provided the evidence that this molecular pair is involved in benign uterine smooth muscle tumours. Further characterization of this molecular pair in its malignant counterpart ULMS may provide a new inside for our understanding of ULMS.

In this study, a seemly inverse association of HMGA2 protein and let-7c expression was observed. Although the global expression pattern of miRNA in ULMS remains to be determined, a loss of let-7 expression was identified in a proportion of ULMS. Particularly, we illustrate that exogenous let-7 can significantly reduce the tumour cell proliferation in leiomyosarcoma cell lines, largely through repression of HMGA2 expression (Figs 3 and 4). Therefore, it is speculated that disrupting of HMGA2 and let-7 pairs in ULMS could be an important molecular change responsible for ULMS growth. In our future study, it will be important to compare whether overexpression of HMGA2 and loss of let-7 are associated with unfavourable clinical outcome and specific histological grade in a large cohort of ULMS cases.

Another interesting finding is that the karyotype characterized by specific chromosome 12 changes, commonly seen in benign ULM, was also identified in one ULMS in our study. Further analysis revealed an overexpression of HMGA2 mRNA and protein in this tumour. The findings raise two questions:

1. Can overexpression of HMGA2 in ULMS be induced through specific chromosome changes involving chromosome 12, at least in a small proportion of tumours? A recent study of CGH in seven ULMS showed that one of the highest levels of genomic gains was in chromosome region 12q13–15 [4], where HMGA2 is located. This study suggested that overexpression of HMGA2 is commonly associated with increase genomic material in this region. Findings of two types of chromosome 12 changes involving different tumour regions in one leiomyosarcoma in this study apparently are not coincidence.

2. Is there a molecular connection between benign and malignant uterine smooth muscle tumours to a non-random chromosome 12 changes? It is generally recognized that ULMS may develop independently from ULM, as it is (i) commonly a solitary mass of uterus, (ii) commonly in the peri- and post-menopausal women and (iii) composed of different genetic alterations as compared to leiomyomas. However, it is not uncommon that ULMS contain usual or atypical leiomyoma-like areas (LLA) [26]. The nature of LLA within ULMS has not yet been established. In the case with
chromosome 12 changes, the reason we selected two different regions for cytogenetic analysis was that this tumour consists of both histological malignant and LLA regions. It remains a challenge to characterize whether the chromosome 12q changes in ULMS arises from the possible LLA area or frankly from well-differentiated ULMS.

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