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HMGI(Y) Expression in Human Uterine Leiomyomata

Involvement of Another High-Mobility Group Architectural Factor in a Benign Neoplasm

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Chromosomal rearrangements involving 6p21 have been observed in uterine leiomyomata and a variety of other benign tumors. The gene for* HMGI(Y)*, a member of the high-mobility group (HMG) family of proteins, has been localized to 6p21. To determine whether rearrangements observed in this area alter* HMGI(Y) *expression, we analyzed HMGI(Y) DNA-binding activity in protein extracts from uterine leiomyoma and normal myometrium tissues. This report describes a uterine leiomyoma specimen with an inv(6)(p21q15). A genomic P1 clone that contains the* HMGI(Y) *region of chromosome 6 is found to span the inversion breakpoint by fluorescent* in situ *hybridization of metaphase chromosomes. Expression of HMGI(Y) protein in this leiomyoma specimen is increased dramatically as compared with the matching normal myometrial tissue. Elevated HMGI(Y) expression was also found in 8 of 16 leiomyomas without cytogenetically detectable chromosome 6p21 aberrations but not in any of the 9 matching myometrial tissues. Analysis of the genetic events involved in the pathobiology of these benign tumors will provide a basis for understanding the process of improper cellular growth and might be important in deciphering the multistep pathway of tumorigenesis. (Am J Pathol 1997, 150:911–918)

Uterine leiomyomata, commonly known as fibroids, are benign smooth muscle cell tumors that are the most frequent indication for hysterectomy. With a prevalence rate estimated to be as high as 77% in females of reproductive age or older,[1] these tumors are associated with a variety of biological sequelae including pelvic pain, abnormal uterine bleeding, and infertility. Uterine leiomyomata are generally detected as multiple, independent nodules of diverse form and size, although they may also be present as solitary tumors. Approximately 40% of these tumors contain chromosomal aberrations such as translocations between 12q14–15 and 14q23–24, deletion or rearrangement of 3q and 7q, abnormalities of 13q, trisomy 12, and rearrangements involving 6p21 and 10q22.[2–4] We and others have shown recently that rearrangements of 12q14–15 in uterine leiomyomata, as well as a variety of other mesenchymally derived benign tumors, involve the architectural factor *HMGI-C*, resulting in aberrant expression or fusion transcripts.[5–8] The gene for HMGI(Y), the other member of the HMGI protein family, has been mapped to the short arm of chromosome 6 at 6p21.[9] Rearrangements involving 6p21 have been observed not only in uterine leiomyomata[10] but also in a variety of benign tumors such as angioleiomyoma,[11] endometrial polyps,[12] and lipomas.[13] A recent study of pulmonary hamartomas by Xiao and co-workers (reported elsewhere in this issue)[14] demonstrates chromosome band 6p21 rearrangements that specifically target the *HMGI(Y)* gene. Based on the finding of rearrangements at 6p21 and the involvement of *HMGI-C* in uterine leiomyomata with 12q14–15 rearrangements, we investigated whether *HMGI(Y)* is also dysregulated in these tu-

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mors. We now report that in a uterine leiomyoma containing an inv(6)(p21q15) the breakpoint occurs adjacent to (but not within) the *HMGI(Y)* gene. This rearrangement is accompanied by HMGI(Y) overexpression in protein extracts from tumor tissue but not from matching myometrial tissue. In addition, expression of HMGI(Y) is detected in 8 of 16 leiomyomas that do not contain cytogenetically detectable 6p21 alterations. Our findings demonstrate ectopic HMGI(Y) expression in uterine leiomyomata and expand the involvement of architectural factors in these benign uterine tumors to include *HMGI(Y)*.

Materials and Methods

Polymerase Chain Reaction Screening of Human P1 Library

Primers were generated from the 5′UT region of the human *HMGI(Y)* gene that could amplify a 323-bp fragment from human genomic DNA by polymerase chain reaction (FP-1, 5′-GACTTGTAT CTCACCAATCAGCCC-3′, and RP-1, 5′-AGAGGAGTCACGAAGGAGCTT-3′). These primers were provided to Genome Systems (St. Louis, MO) for screening the DuPont Merck Pharmaceutical Company Human Foreskin Fibroblast P1 Library by polymerase chain reaction. A positive clone was identified from dish 1073, row D (P1 clone 6995). DNA was isolated from the P1 clone as recommended by the company and was used for restriction enzyme digests and Southern blot analysis to map the location of the *HMGI(Y)* gene within the clone.

Tumor Cell Lines and Chromosome Preparations

Leiomyomata and myometrial specimens were obtained from patients at the time of surgery. Tumor culture, metaphase chromosome harvesting, slide preparation, and trypsin-Giemsa banding were performed as described previously.[15]

In Situ *Hybridization*

P1 clone 6995 was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions using 1 μg of the P1 DNA, dNTPs obtained from Boehringer Mannheim, and the DNAse I/DNA polymerase I mix from the BioNick labeling kit (Gibco BRL, Gaithersburg, MD). Metaphase spreads were pretreated in 2X standard saline citrate (SSC) at 37°C for 30 minutes and dehydrated in 70, 80, 90, and 100% ethanol for 2 minutes each before denaturation in 70% formamide at 70°C for 1 minute, 45 seconds, and dehydration again in ice-cold 70, 80, 90, and 100% ethanol. The digoxigenin-labeled P1 clone was diluted to 10 μg/ml with Hybrisol VI (Oncor, Gaithersburg, MD) and then denatured at 70°C for 5 minutes. A 10-μl aliquot of denatured probe was placed onto each slide, which was then sealed with a coverslip and rubber cement, and incubated at 37°C for 16 hours. Slides were washed in 60% formamide at 43°C for 15 minutes and then in 2X SSC at 43°C for 10 minutes. Probe was detected using a rhodamine-labeled anti-digoxigenin detection kit (Oncor) and chromosomes were counterstained with 4,6-diamidino-2-phenylindole-dihydrochloride. Hybridization signals were observed using a Zeiss Axioskop microscope and images were captured with the CytoVision imaging system (Applied Imaging, Pittsburgh, PA).

Extract Preparation

Frozen tissue samples were minced with a scalpel blade and then placed into twice their volume of buffer C (20 mmol/L Hepes, pH 7.9, 1.5 mmol/L $MgCl_2$, 420 mmol/L NaCl, 0.2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, 1.5 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.04 mmol/L N-Ac-Leu-Leu-Nle, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride). Samples were vortexed, refrozen on dry ice, and thawed a total of three times, and then spun at 14,000 rpm for 30 minutes at 4°C. Supernatants were removed and stored at −80°C.

Oligonucleotide Synthesis and Radiolabeling

Oligonucleotides were synthesized using a 392 DNA synthesizer (Applied Biosystems, Foster City, CA) and were purified by polyacrylamide gel electrophoresis. Double-stranded probes were generated by annealing complementary oligonucleotides and filling in overhanging ends with 50 μCi [α-^{32}P]dCTP (3000 Ci/mmol, New England Nuclear, Boston, MA) and DNA polymerase I, Klenow fragment (New England Biolabs, Beverly, MA). Unincorporated nucleotides were removed using Chromaspin-10 columns (Clontech, Palo Alto, CA).

Electrophoretic Mobility Shift Assay (EMSA)

Binding reactions were carried out in a total volume of 20 μl containing approximately 13 μg of tissue protein

Table 1. *Karyotype of Uterine Leiomyomata and Semiquantitative Analysis of HMGI(Y) Binding Activity*

Case number	Specimen number	Specimen	Karyotype	HMGI(Y) binding	Tumor size (cm)	Patient age
1	ST92-224*	UL	46,XX,t(1;12)(q32;q14),del(7)(q22q32)	−	7.5	34
	ST92-225	M	ND	−		
2	ST93-054	UL	46,XX	+	5.5	38
	ST93-055*	UL	45,XX,t(1;7)(q31;q22),−2,−3,−6,add(11)(q21),18,+mar1	+	3.5	
	ST93-056	UL	46,XX	−	2.5	
	ST93-057	M	ND	−		
3	ST93-165†	UL	46,XX,del(1)(q42),t(12;14)(q14;q24)	−	7.5	52
	ST93-166	M	ND	−		
4	ST93-219	UL	46,XX	+	6.0	40
	ST93-220†	UL	46,XX,t(12;14)(q13;q32)	−	3.0	
	ST93-221	M	ND	−		
5	ST93-397	UL	46,XX,inv(6)(p21q15),inv(9)	++++++	13.5	43
	ST93-398	M	ND	−		
6	ST93-731	UL	46,XX	++	9.5	33
	ST93-732	UL	46,XX	−	3.0	
	ST93-734	M	ND	−		
7	ST93-738†	UL	46,XX,t(12;14)(q14-15;q23-24)	−	14.5	56
	ST93-739	M	ND	−		
8	ST94-008	UL	45,XX,del(1)(p22p35),−6,add(10)(q22),19,+marl	−	9.0	47
	ST94-009	UL	46,XX	−	5.0	
	ST94-010	M	ND	−		
9	ST94-143	UL	46,XX	+	7.0	34
	ST94-144	UL	46,XX	+	7.0	
	ST94-146‡	UL	46,XX,del(7)(q11),der(14)t(7;14)(q22;q32)[11]/46,XX[6]	+++	7.0	
	ST94-147	M	ND	−		
10	TC95-1	UL	ND	+++	8.4	49
	TC95-2	M	ND	−		

UL, uterine leiomyoma; M, myometrium. Each UL specimen was taken from an independent tumor in cases in which a patient had multiple fibroids. ND, not determined; −, no detectable HMGI(Y) binding. Quantitative analysis of positive HMGI(Y) binding activity was determined by comparing the relative intensities of DNA-protein complexes from EMSA autoradiographs and by comparing the weights (in mg) of shifted bands when cut out from a copy of the autoradiograph.

*Karyotypes have been previously reported.[34]

†Karyotypes have been previously reported.[8]

‡Karyotype has been previously reported.[35]

extract (except for samples ST93–397 and ST93–398 for which only 4 μg of protein extract was used due to the high level of HMGI(Y) binding activity in this tumor), 0.2 μg of poly (dG·dC)-poly (dG·dC) (Sigma Chemical Co., St. Louis, MO) and 100,000 cpm ^{32}P-labeled oligonucleotide probe in 10 mmol/L Tris, pH 7.5, 50 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, and 5% glycerol. Reactions were allowed to continue for 20 minutes at room temperature and were then loaded onto 5% nondenaturing polyacrylamide gels that were run at 200 V (constant voltage) in 1X Tris Borate-EDTA buffer for 1.5 hours. Gels were dried under vacuum and autoradiographed overnight (with the exception of Figure 2B, which was exposed to film for only 7 hours) using Kodak X-OMAT-AR film.

Results

We identified a uterine leiomyoma (ST93–397) with a 46,XX, inv(6)(p21q15), inv(9) (Figure 1A, inset) from cytogenetic analysis of a series of leiomyomata. To assess whether the inversion breakpoint of leiomyoma ST93–397 at 6p21 is in close proximity to the *HMGI(Y)* gene, fluorescence *in situ* hybridization was performed on metaphase chromosomes using a human genomic P1 clone (6995) that contains the *HMGI(Y)* gene (Figure 1B). This clone gave a hybridization signal on the short arm of the normal chromosome 6 and on both the short and the long arms of the inverted chromosome 6 (Figure 1A). This result suggests that the inversion breakpoint is contained within the sequence in the P1 clone. Southern blot analysis of DNA from this leiomyoma digested with multiple restriction enzymes revealed bands consistent with its genomic structure, indicating that the *HMGI(Y)* gene is intact (data not shown). The total length of the P1 clone is not more than approximately 75 kb; thus, it is possible for the inversion breakpoint in 6p21 to be close enough to the *HMGI(Y)* gene to have altered the normal cellular regulation of *HMGI(Y)*.

The ability of HMGI(Y) to bind specifically to an element in the interferon-β1 (*IFN-β*) promoter, designated positive regulatory domain II (PRDII),[16] was used to analyze uterine leiomyoma and myometrial protein extracts for HMGI(Y) DNA-binding activity.

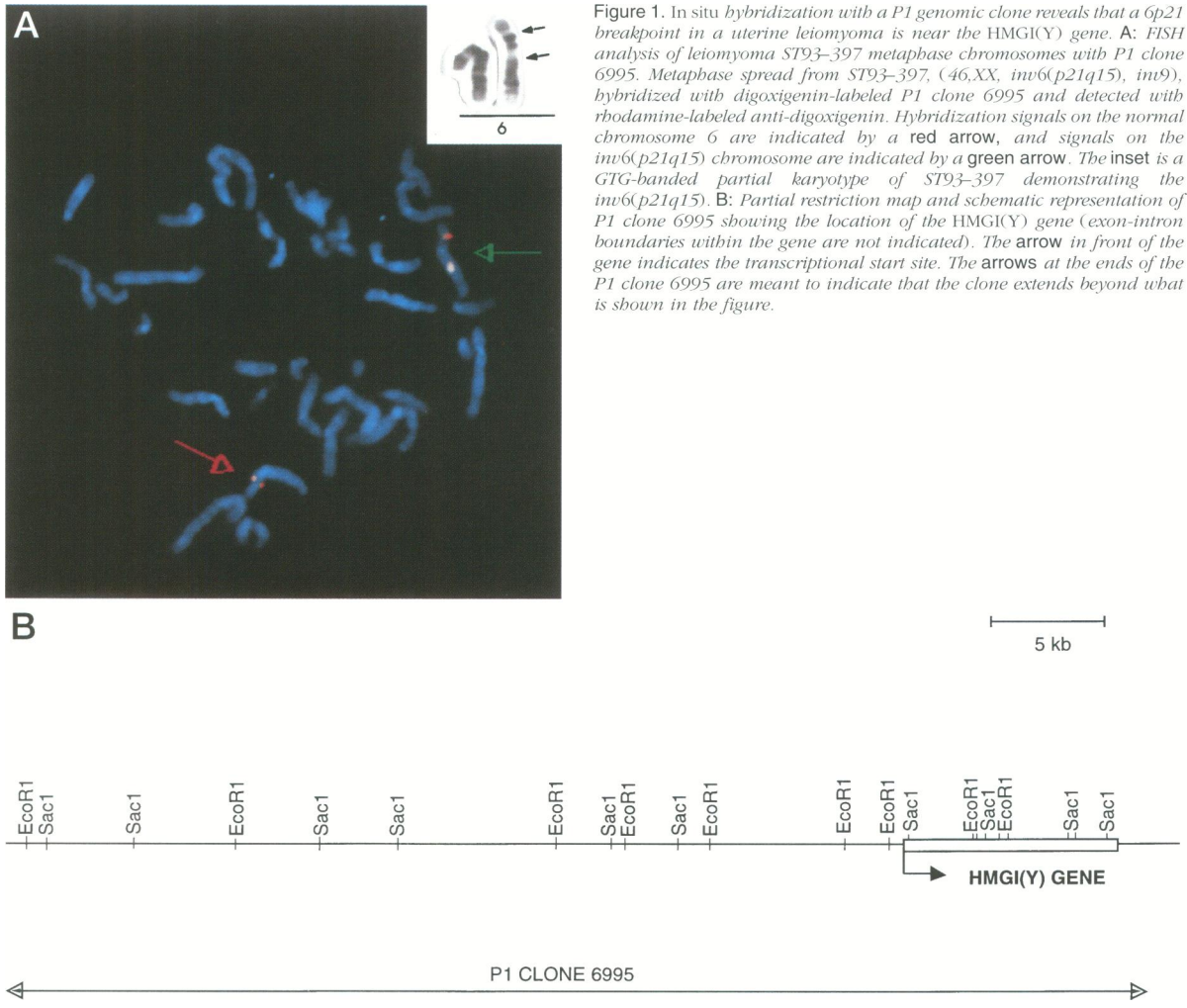

Figure 1. In situ *hybridization with a P1 genomic clone reveals that a 6p21 breakpoint in a uterine leiomyoma is near the* HMGI(Y) *gene.* A: *FISH analysis of leiomyoma ST93–397 metaphase chromosomes with P1 clone 6995. Metaphase spread from ST93–397, (46,XX, inv6(p21q15), inv9), hybridized with digoxigenin-labeled P1 clone 6995 and detected with rhodamine-labeled anti-digoxigenin. Hybridization signals on the normal chromosome 6 are indicated by a* red arrow, *and signals on the inv6(p21q15) chromosome are indicated by a* green arrow. *The* inset *is a GTG-banded partial karyotype of ST93–397 demonstrating the inv6(p21q15).* B: *Partial restriction map and schematic representation of P1 clone 6995 showing the location of the* HMGI(Y) *gene (exon-intron boundaries within the gene are not indicated). The* arrow *in front of the gene indicates the transcriptional start site. The* arrows *at the ends of the P1 clone 6995 are meant to indicate that the clone extends beyond what is shown in the figure.*

Total protein extract was prepared from leiomyoma ST93–397 as well as from normal myometrium from the same patient (ST93–398). In an EMSA with a labeled oligonucleotide probe (PRDII X 2, Figure 2A), protein extract from ST93–397 gave a low molecular weight gel shift band whereas no such band was seen with extract from ST93–398 (Figure 2B). To confirm the identity of the band as HMGI(Y), an oligonucleotide was synthesized that altered the HMGI(Y) recognition site (mPRDII X 2, Figure 2A). This mutation was previously shown to disrupt HMGI(Y) binding while not affecting binding of nuclear factor-κB (NF-κB).[16] When the mutated probe was used instead of the intact site, the shifted band seen with ST93–397 extract was almost completely abolished, whereas binding of recombinant p50 homodimer (a form of NF-κB)[17] was not affected (Figure 2B). Additionally, when poly (dI-dC) was substituted for poly (dG-dC) as a nonspecific competitor, HMGI(Y) binding was completely eliminated (data not shown) as previously described.[18] The loss of binding activity when the HMGI(Y) binding site is mutated and the competition by poly (dI-dC) strongly suggest that the shifted complex is HMGI(Y).

Protein extracts were also prepared from 16 randomly selected leiomyomata of various karyotypes along with matching myometrial samples from 9 different patients (Table 1) for gel shift analysis with the HMGI(Y) DNA-binding probe PRDII X 2. Although HMGI(Y) DNA-binding activity was not detected in any of the 9 matching myometrial control tissues, it was readily detected in 8 of the 16 tumors analyzed (Figure 3). Karyotypes of all but one of the 16 tumor samples were determined and none was found to have any microscopically visible rearrangement of chromosome 6 (Table 1). These results demonstrate that ectopic expression of *HMGI(Y)* can occur in the absence of cytogenetic abnormalities, suggesting that these tumors may have acquired small mutations, undetectable by standard cytogenetic tech-

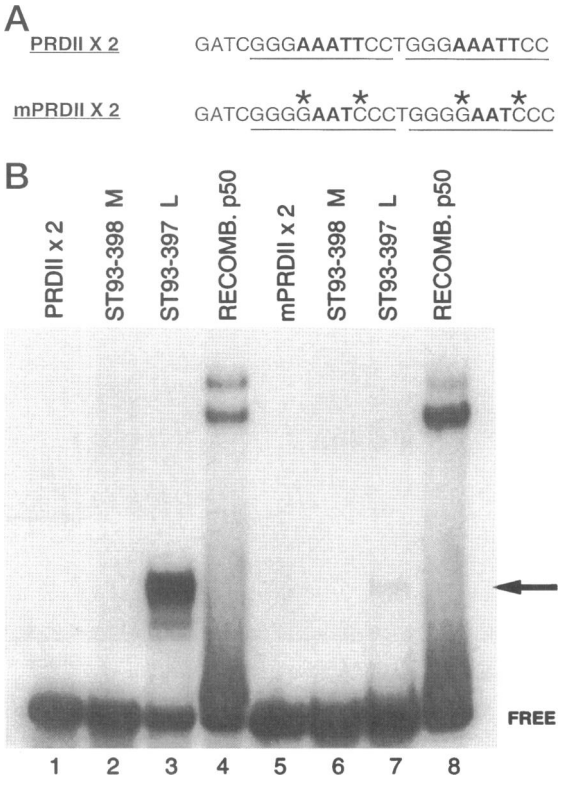

Figure 2. *HMGI(Y) DNA-binding activity is increased in a uterine leiomyoma with an inv6(p21q15).* **A:** *Sequence of oligonucleotides PRDII X 2 and mPRDII X 2 used in EMSA analysis. The duplicated NF-κB site (PRDII)*[16] *from the human IFN-β promoter has been underlined. Bolded nucleotides show the HMGI(Y) binding site and asterisks above the mPRDII X 2 sequence indicate those nucleotides that were changed to mutate the binding site.* **B:** *EMSA analysis of protein extract from leiomyoma ST93–397.* **Lanes 1** *to* **4** *contain* ^{32}*P-labeled PRDII X 2 oligonucleotide probe mixed with no extract* (**lane 1**), *4 μg of total protein extract from normal myometrium tissue ST93–398* (**lane 2**), *4 μg of total protein extract from leiomyoma tissue ST93–397* (**lane 3**), *and recombinant p50* (**lane 4**). **Lanes 5** *to* **8** *contain* ^{32}*P-labeled mPRDII X 2 mixed with no extract* (**lane 5**), *4 μg of total protein extract from normal myometrium ST93–398* (**lane 6**), *4 μg of total protein extract from leiomyoma ST93–397* (**lane 7**), *and recombinant p50* (**lane 8**). *Recombinant p50 homodimer (a form of NF-κB) was used to demonstrate the integrity of both wild-type and mutated PRDII X 2 oligonucleotides. Bound complexes are indicated by an* **arrow** *to the right of the figure. FREE indicates the location of unbound probe.*

niques. Notably, the amount of binding activity varied between fibroids, including those obtained from the same individual. Four patients showed no expression of HMGI(Y) in either tumor or myometrial tissue, whereas six patients had measurable expression in at least one tumor. The highest amount of HMGI(Y) binding activity was seen in ST93–397, the tumor that had an inv(6)(p21q15). Only 4 μg of protein from tumor sample ST93–397 was required to generate the shifted band in Figure 2B whereas 13 μg of protein was used for each of the samples in Figure 3. No significant correlation was observed between HMGI(Y) binding activity and tumor size or patient age. The variability in HMGI(Y) binding activity may reflect the specific type of mutation that has occurred in each tumor. The difference in levels of HMGI(Y) might also be explained by mosaicism; if the mutation was an early or even a primary event in the tumor, then a greater proportion of cells would express HMGI(Y) than if the mutation occurred much later during tumor development.

Discussion

HMGI(Y) is an important architectural component of higher order chromatin complexes and may play a role in normal transcriptional regulation. Previous investigations have shown that HMGI(Y) binds directly to specific A-T-rich elements in the promoters of several genes including *IFN-β*,[16,19] E-selectin,[20] interleukin-2 receptor α-chain,[18] the chemokine MGSA/GRO,[21] and the class II major histocompatibility complex gene HLA-DRA.[22] In all cases, HMGI(Y) binding is necessary for inducible gene expression. Studies performed with the *IFN-β* promoter have identified at least three HMGI(Y) binding sites; one site is located within the center of a NF-κB binding element and has been designated positive regulatory domain II (PRDII) whereas the other two sites flank an activating transcription factor-2 (ATF-2)/c-jun binding site known as PRDIV.[19] Binding of HMGI(Y) to these sites through contacts in the minor groove facilitates the binding of NF-κB and ATF-2/c-jun to PRDII and PRDIV, respectively, to contacts in the major groove. This binding induces a conformational change in the DNA[23] that allows the transcription factors to interact with each other as well as with the basal transcription complex. Although HMGI(Y) cannot activate transcription on its own,[16] its presence results in a 10-fold increase in the level of expression of the *IFN-β* gene when the transcriptional activators NF-κB and ATF-2/c-jun are also present.[24] Thus, HMGI(Y) facilitates assembly of a stereospecific transcriptional enhancer complex. Furthermore, HMGI(Y) has also been shown to suppress transcription of several genes such as interleukin-4[25] and the immunoglobulin ε-heavy-chain gene[26]; the mechanism by which HMGI(Y) represses these genes has not been elucidated. Inappropriate expression of *HMGI(Y)* in uterine leiomyomata might therefore facilitate activation or repression of a variety of genes relevant to tumor growth and biology.

Elevated levels of architectural proteins may also have an effect on chromatin structure. In histone H1-depleted chromatin, HMGI(Y) is enriched and can mediate displacement of H1 from chromatin in

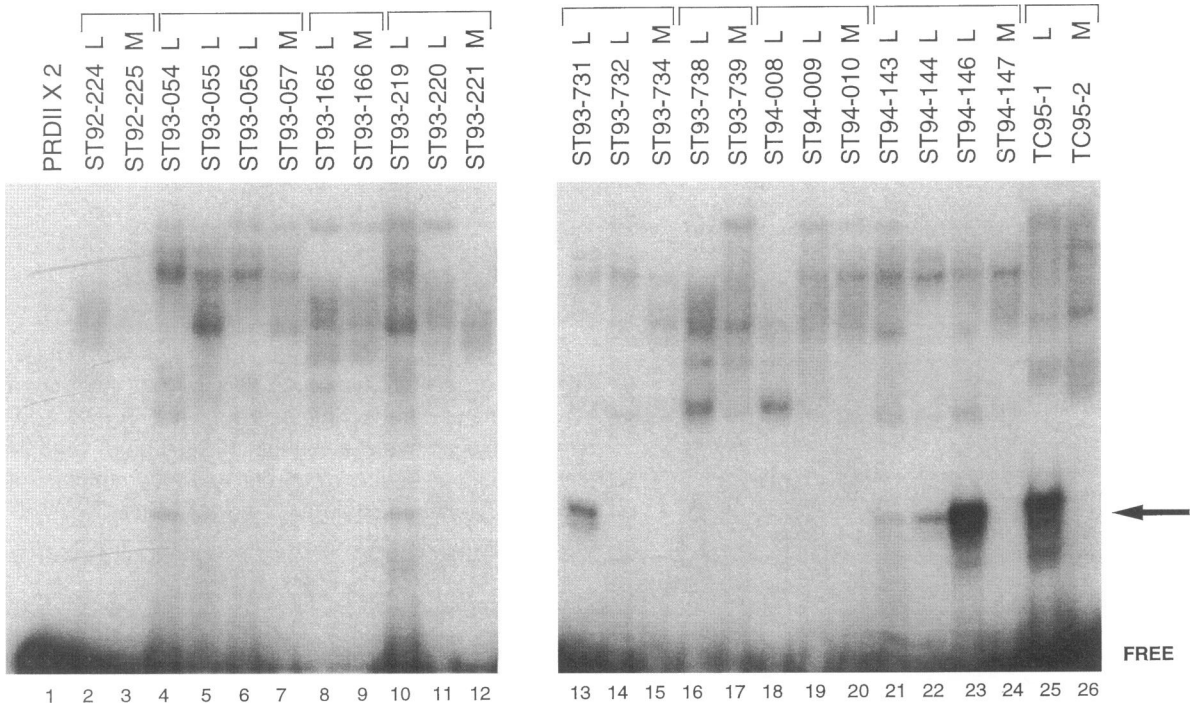

Figure 3. *EMSA analysis of protein extracts from 16 uterine leiomyomas and 10 matched myometrial specimens.* Lanes *contain* ^{32}*P-labeled PRDII X 2 oligonucleotide probe mixed with no protein extract* (lane 1) *or 13 μg of total protein extract from leiomyoma or myometrium* (lanes 2 *to* 26). *Leiomyoma specimen numbers are followed by the letter L whereas myometrium specimen numbers are followed by the letter M. Brackets above the specimen numbers indicate that the tissue samples were obtained from the same patient. The* arrow *to the right of the figure identifies DNA-protein complexes that can be abolished by substituting the oligonucleotide probe mPRDII X 2 in the EMSA reaction. FREE indicates the position of unbound probe.*

scaffold-associated regions.[27] The presence of gaps in the nucleosomal array is frequently associated with the presence of regulatory regions in actively transcribed genes.[28] Increased expression of the architectural proteins may help overcome nucleosomal barriers to transcription or maintain certain chromatin regions in a conformation accessible to other transcriptional activators. Increased expression of HMGI(Y) may also influence the long-range organization of chromatin fibers and thereby affect gene regulation.

Increased levels of HMGI(Y) have been correlated with a malignant phenotype in a variety of tumors such as mouse lung and thymus,[29] mouse mammary epithelial cell,[30] high-grade human prostate tumors,[31] human thyroid carcinoma,[32] and human colorectal carcinomas.[33] Interestingly, uterine leiomyomata are benign tumors that rarely, if ever, become malignant. Therefore, it is particularly intriguing that 50% of the uterine leiomyomata analyzed in this report showed some level of HMGI(Y) expression. Given the putative role of HMGI(Y) as a facilitator of transcription factor binding and activity, perhaps the critical determinant of malignant potential is not solely expression of *HMGI(Y)* but rather the concurrent presence of specific transcription factors (either endogenous or due to additional mutations in other genes), the activities of which can be enhanced by the actions of HMGI(Y). It is possible that the smooth muscle cells of uterine leiomyomata do not express a particular transcriptional activator that interacts with HMGI(Y) to augment expression of a gene(s) required for malignant transformation. Or equally possible, uterine smooth muscle cells may contain a transcription factor responsible for activating expression of a tumor suppressor gene, and this activation is enhanced by HMGI(Y) or HMGI-C, thus maintaining these tumors in a benign state. Whatever mechanism(s) are elucidated, the presence of increased levels of HMGI(Y) and HMGI-C in both benign and malignant tumors suggests that architectural proteins play an important in role in the pathobiology of these disorders.

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